Molecular systematics and phylogeography of the Helmeted Guineafowl (*Numida meleagris*)

by

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Chapter 1

General Introduction

General Introduction

The Helmeted Guineafowl, *Numida meleagris* (Linnaeus, 1758), is the most common and widespread African gamebird (Figure 1.1), occurring in all sub-Saharan open-country vegetation, from forest edge to sub-desert steppe (Little 1997) with the West African subspecies (*N. m. galeata*) having been domesticated throughout much of the world (Del Hoyo *et al.* 1994). This species is a large, gregarious, dark guineafowl with a bluish bare head and neck, topped with a horn-like casque. There are nine recognised subspecies of *N. meleagris*, namely: *N. m. meleagris* (Linnaeus, 1758), *N. m. sabyi* (Hartert, 1919), *N. m. galeata* (Pallas, 1767), *N. m. somaliensis* (Neumann, 1899), *N. m. reichenowi* (Ogilvie-Grant, 1894), *N. m. mitrata* (Pallas, 1767), *N. m. marungensis* (Schalow, 1884), *N. m. damarensis* (Roberts, 1917), and *N. m. coronata* (Gurney, 1868). The Moroccan subspecies *N. m. sabyi* has recently become very rare, with only three records in the 1970s (Del Hoyo *et al.* 1994). The subspecies are distinguished from one another by the variation in head colour and adornments as well as casque shape (Figure 1.1) (Crowe *et al.* 1986).

The Helmeted Guineafowl is a member of the family Numididae (Guineafowl), which belongs to the order Galliformes. All members of the family are medium sized (40–72 cm tall), plump, terrestrial birds with bare heads and necks (with the bare skin often brightly coloured), with most species having feathers or a bony casque on the crown (Crowe *et al.* 1986). The guineafowl have formerly been considered a subfamily (Numidinae) of Phasianidae but studies based on DNA-DNA hybridisation suggested that family treatment is appropriate for the group (Sibley *et al.* 1988) and it was estimated that the Numididae diverged from the Phasianidae lineage some 38 million years ago (Sibley and Ahlquist 1990). A recent study based on cytochrome *b* and ND2 sequence data dates this split at 50-54 million years ago (Van Tuinen and Dyke 2004). The closest relatives of the Numididae are apparently to be found in Phasianidae and Cracidae (Cracraft 1981; Sibley and Ahlquist 1990). The changes in the distribution patterns of vegetation over geological time, determined by climatic fluctuations, would have divided biomes into more or less isolated areas providing the opportunity for guineafowl to diverge in isolation. This in turn has led to the various representatives of the family occupying practically all of the biotopes found in Africa in the present day (Crowe 1978).

The Numididae is a well-defined endemic African family comprising four genera: *Agelastes*, *Guttera*, *Acryllium* and *Numida*. Within these genera are six species and 19 subspecies (one of these species is threatened and possibly extinct). All four of the genera currently recognised within the family were clearly differentiated by the Pleistocene (Del Hoyo *et al.* 1994), approximately 1.75 million years ago. Of the four, *Agelastes* is probably the most primitive, with its two species constituting a superspecies (Del Hoyo *et al.* 1994).

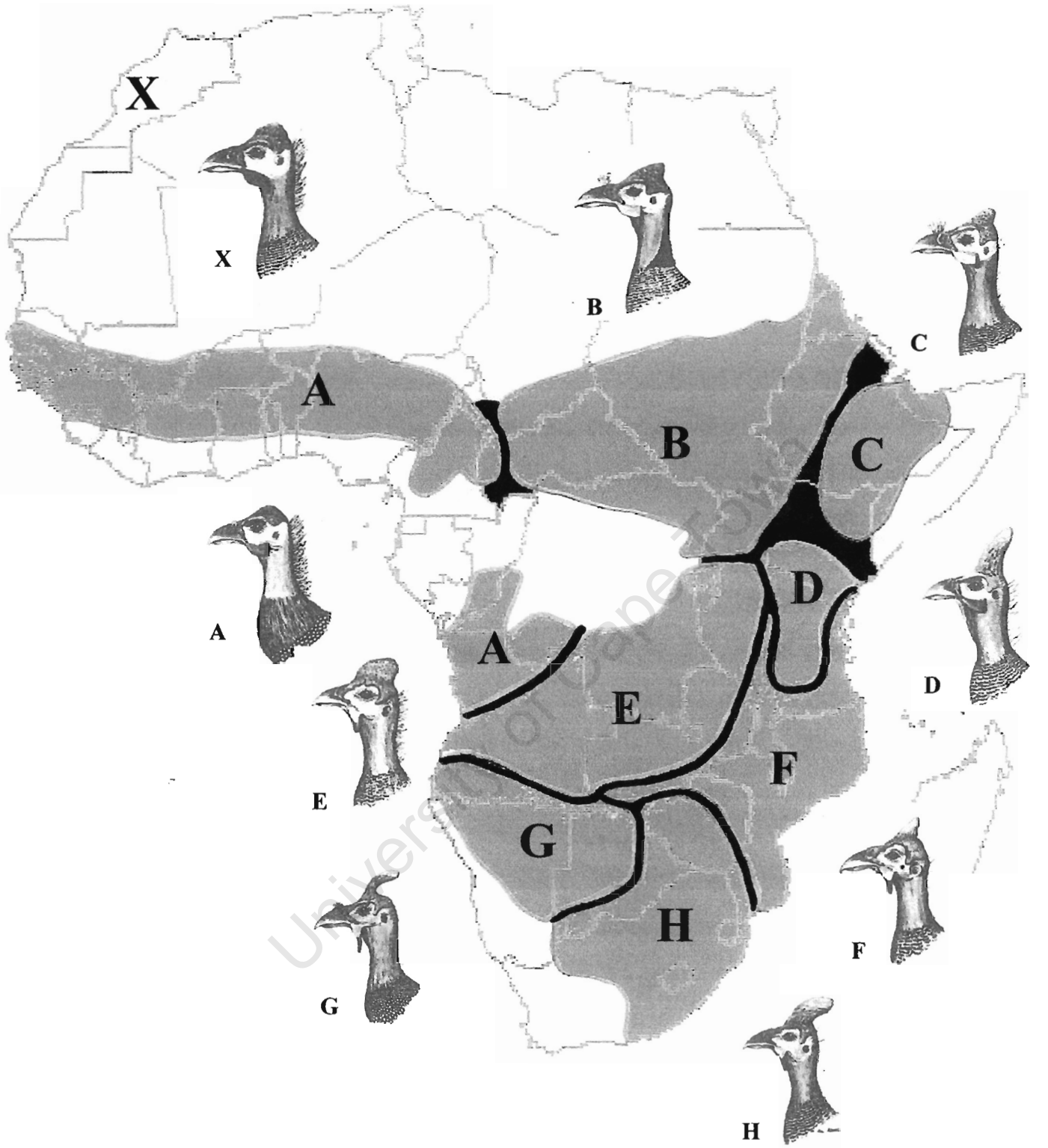


Figure 1.1. Helmeted Guineafowl subspecies and their distributions. A-*Numida meleagris galeata*, B-*N. m. meleagris*, C- *N. m. somaliensis*, D- *N. m. reichenowi*, E- *N. m. marungensis*, F- *N. m. mitrata*, G- *N. m. damarensis*, H- *N. m. coronata*, X- *N. m. sabyi*. (Map modified from Crowe & Snow, 1978; guineafowl illustrations reproduced from Crowe, 1978)

Members of the family

Genus *Agelastes* (Bonaparte, 1850)

There are two monotypic species in the genus, *A. meleagrides* and *A. niger*, with very little known about either of them. They are small guineafowl with dull to bright pink skin and have no plumes or tufts on their crown. The body plumage is black, or black with a white collar with varying amount of white vermiculation (Crowe *et al.* 1986). *Agelastes* species are the most primitive members of the family, most closely related to an ancestral francolin-like stock (Ghigi 1936; Crowe 1978), or perhaps from the same stock that gave rise to the junglefowl.

Agelastes meleagrides (Bonaparte, 1850) (Figure 1.2), the White-breasted Guineafowl, is resident in primary forests of West Africa from Liberia to Ghana. It is distinguished from other forest guineafowl (*Guttera*), which have bright blue spotted plumage and crested heads with slaty grey naked skin, by a bare, bright reddish head and conspicuous white collar and breast contrasting sharply with dark body plumage. The White-breasted Guineafowl is possibly one of the most endangered birds in Africa, and is severely threatened by hunting pressure and habitat destruction and will probably disappear except from a few protected areas (Del Hoyo *et al.* 1994).

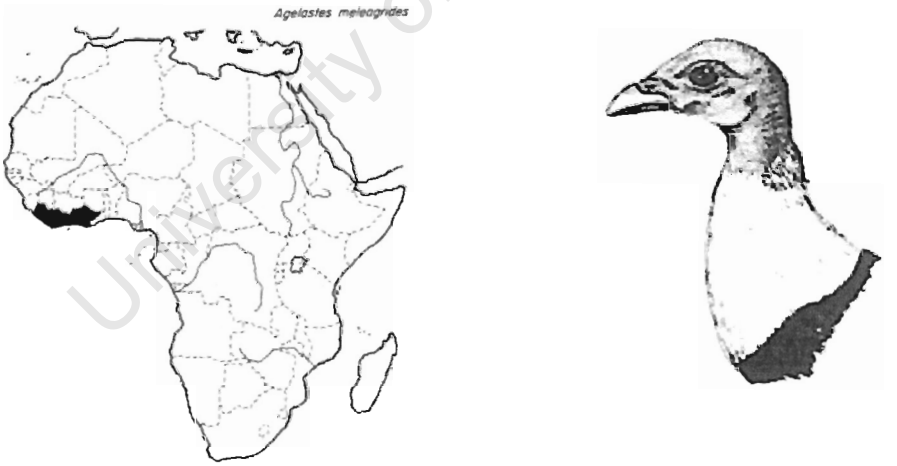
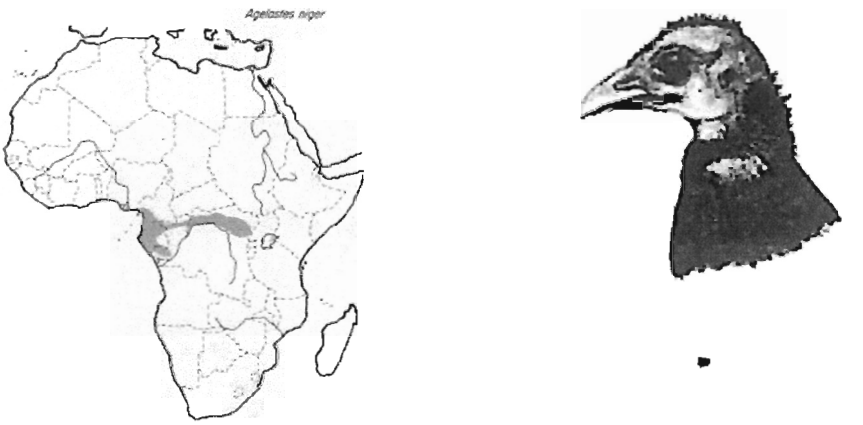


Figure 1.2. Distribution and morphology of *A. meleagrides* (distribution from Crowe *et al.* 1986, guineafowl illustration taken from Crowe 1978)

The Black Guineafowl, *A. niger* (Cassin, 1857) (Figure 1.3), inhabits dense primary forests from Cameroon and Gabon south to the lower Congo basin eastwards north of the western rift valley. It is a small black guineafowl with a short crest of downy black feathers. Like the White-breasted Guineafowl it is scarce, but probably less threatened by habitat destruction than *A. meleagrides* because its range is much more extensive (Hastings Belshaw 1985).



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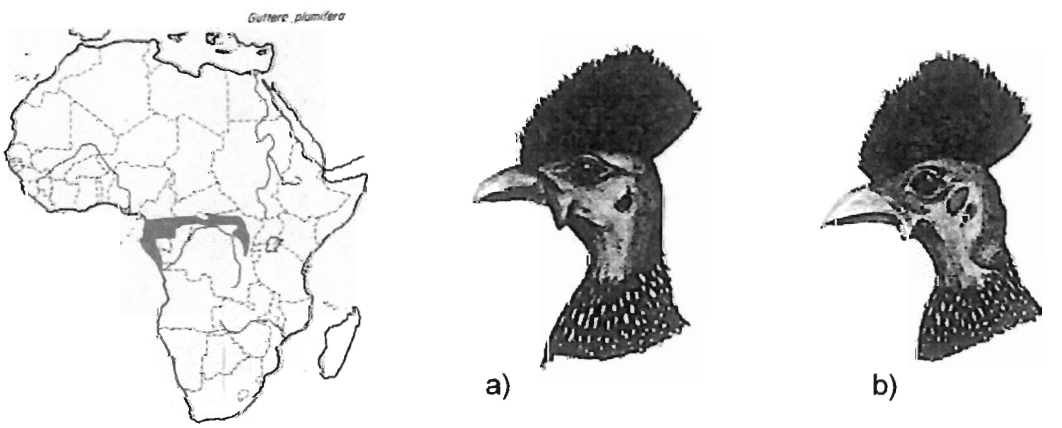
Genus *Guttera* (Wagler, 1832)

The genus has been divided into three species, *G. plumifera*, *G. pucherani* and *G. edouardi* but Ghigi (1936) and Crowe (1978) regard the latter pair as conspecific based on intergradation in captivity and in the wild. Since they are considered to be conspecific the principle of priority is followed and the specific name recognised is *G. pucherani* (Hartlaub, 1860). It is a rather large guineafowl, with a naked head and neck largely dull blue-grey with a crest of long black feathers. Members of the genus *Guttera* inhabit forest and dense scrub (Del Hoyo *et al.* 1994).

Guttera plumifera (Cassin) (Figure 1.4), the Plumed Guineafowl, is resident in dense forests from South Cameroon to lower DRC, inland in forests of the Congo basin and east to the western edge of the western rift valley. It is uncommon and probably not uniformly distributed within this range. There are two described subspecies, *G. p. plumifera* (Cassin, 1857) and *G. p. schubotzi* (Reichenow, 1912) (Crowe *et al.* 1986).



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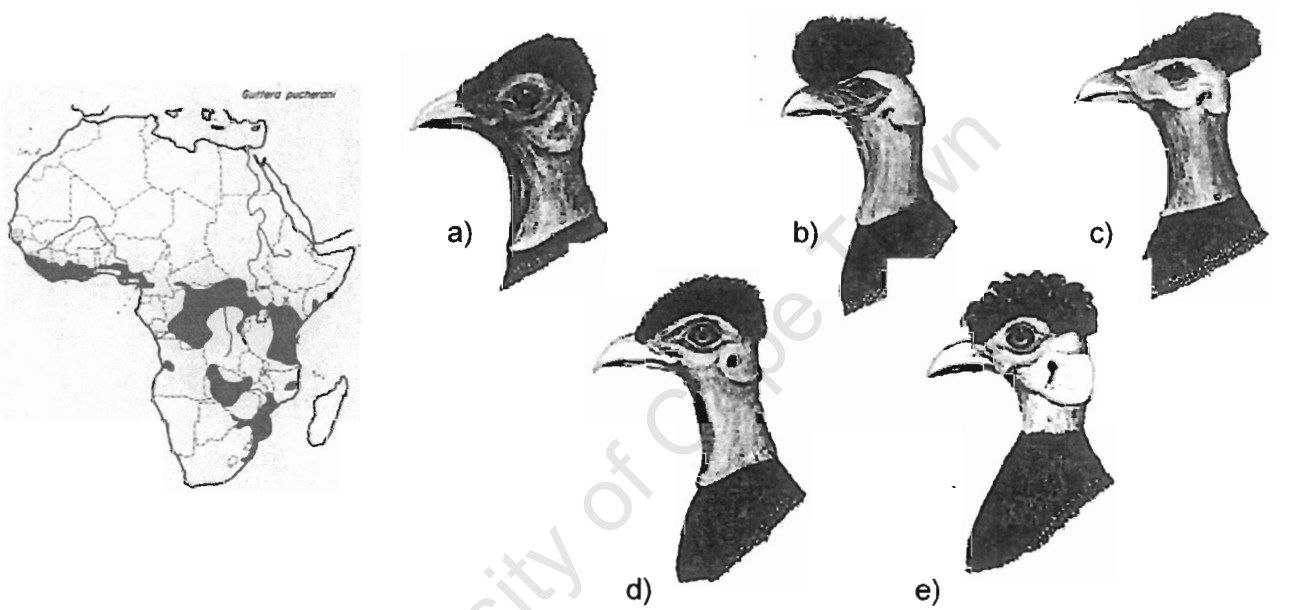
The Crested Guineafowl, *G. pucherani* (Hartlaub, 1860) (Figure 1.5), is resident from Guinea-Bissau east through all forested areas to southern Somalia, south to KwaZulu-Natal in South Africa and in denser *Brachystegia* woodlands with underlying evergreen thickets in Angola and Zambia. Of the many races described only five are recognised as subspecies, they differ in colour of the bare skin and shape and extent of the feather crest. They are: *G. p. pucherani* (Hartlaub, 1860), *G. p. verreauxi* (Elliot, 1870) (including former *seth-smithi*, *schoutedeni*, *pallasi*, *chapini*, *kathleenae*), *G. p. sclateri* (Reichenow, 1898), *G. p. barbata* (Ghigi, 1905) and *G. p. edouardi* (Hartlaub, 1867) (Crowe *et al.* 1986; Del Hoyo *et al.* 1994).



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Genus *Acryllium* (Gray, 1840)

The genus *Acryllium* is monotypic and confined to Somalia and the East African arid region. It is the largest and most brilliantly coloured of the guineafowl, with basic spotted plumage highlighted with iridescent blues, lilac and white (Hastings Belshaw 1985).

The Vulturine Guineafowl, *A. vulturinum* (Hardwicke, 1834) (Figure 1.6), is resident from extreme north east Uganda, north to southern Ethiopia, Somalia, arid parts of northern and eastern Kenya, south to the Pangani river in Tanzania. It inhabits semi-arid *Acacia/Commiphora* scrub, often with shrubs and enters montane forest and forages in tall riverine *Acacia* woodland. It is also known to enter very dense thickets when it occurs with crested guineafowl (Crowe *et al.* 1986).

Figure 1.6. Distribution and morphology of *A. vulturinum* (distribution from Crowe *et al.* 1986, guineafowl illustration taken from Crowe 1978)

The genus *Numida* (Linnaeus 1766) has been described above with the distribution and morphological characteristics of the nine subspecies presented in Figure 1.1.

Systematics of *Numida meleagris*

Over the years the Helmeted Guineafowl has been subdivided into some 30 subspecies but at present only nine are generally recognised (Crowe 1978; Sibley and Monroe Jr 1990) (Figure 1.1). Two or sometimes three of these races have actually been considered separate species by some authors but intergradation in the wild suggests that they are all conspecific (Crowe 1978). While there is only limited support for this approach, there are three fairly clearly defined subgroups: The nominate form in East Africa, the *galeata* group in West Africa and the *mitrata* group in southern Africa (Hastings Belshaw 1985; Del Hoyo *et al.* 1994).

N. m. meleagris (Figure 1.1B) (including former *major, inermis, omoensis, macroceras, neumanni, toruensis, intermedia, uhehensis*): occurs from eastern Chad, east to Ethiopia and south to the northern borders of the Democratic Republic of the Congo, Uganda and northern Kenya. The only populations south of the equator occur in Uganda (Crowe *et al.* 1986). The head and neck are bare but the lower hindneck is covered by short, dense, black filoplumes, grading into a broad greyish black collar finely barred with white. The casque is red-brown at the base grading to horn colour at the apex. The rest of the upperparts, including the upperwing and tail-coverts are dark grey to black with white spots interspersed with a network of white vermiculations. The tail is a dull black, heavily spotted and vermiculated with white. The cere is covered with dense cartilaginous bristles up to 6mm long. The cheeks, area around the eye and most of neck is grey-blue and the hindneck black. The gape wattles are round and blue, with the eye dark brown and the legs dark grey to black (Del Hoyo *et al.* 1994).

N. m. sabyi (Figure 1.1X) (probably now extinct) inhabits northwest Morocco between the Oum er Rbia and Sebou rivers (Crowe *et al.* 1986). It is like *meleagris* but the facial skin is a pale blue-white, there are no cere bristles, the wattles are red, the hindneck filoplumes are very long and confined to the mid-line, and the collar is violet-grey (Del Hoyo *et al.* 1994).

N. m. galeata (Figure 1.1A) (including former *marchei, callewaerti, blancoui*): occurs from West Africa east to southern Chad and south to the central Democratic Republic of the Congo and northern Angola. It is like *sabyi* except the collar is greyish and the casque is smaller, with a distinctive white face and rounded, entirely red wattles (Crowe *et al.* 1986). The plumage of domesticated birds derived from this race is white or a mixture of wildtype and white, with legs orange and not grey or black. Domesticated birds weigh 30-50% more than wild birds (Del Hoyo *et al.* 1994).

N. m. somaliensis (Figure 1.1C) inhabits arid parts of north-eastern Ethiopia and Somalia. It is similar to *meleagris* but the cere bristles are very long (up to 24 mm), the hindneck filoplumes are very long and restricted to the mid-line, and the wattles are somewhat pointed (not rounded) and blue with red tips (Crowe *et al.* 1986).

N. m reichenowi (Figure 1.1D) (including former *ansorgei*) occurs in Kenya and central Tanzania. It is also similar to *meleagris* but the facial skin is a pale blue-white, the wattles are entirely red and rounded, there is a much longer spear-shaped casque, the hindneck filoplumes are long and confined to the mid-line, and there is much less vermiculation of the wing, body and tail feathers (Crowe *et al.* 1986).

N. m. mitrata (Figure 1.1F) is found in coastal and western Tanzania south to coastal Mozambique, west through the Zambezi valley and Zimbabwe to southern Angola and northern Botswana. It is like *reichenowi* but the casque is smaller (but still larger than that of *meleagris*), the wattles are pointed (not rounded) and blue with red tips, and the facial skin is blue-grey (Crowe *et al.* 1986).

N. m. marungensis (Figure 1.1E) (including former *maxima, frommi, rikwae, bodalyae*) occurs in central African savannas and woodlands south of the Zaire basin, south to western Angola and east in the Zambezi Basin to the Luangwa Valley in Zambia. It differs from *mitrata* in having a large, yellow bulbous helmet and pennant shaped, blue wattles with red tips (Crowe *et al.* 1986).

N. m. damarensis (Figure 1.1G) (including former *papillosa*) inhabits the arid parts of Botswana and Namibia. It is similar to *mitrata* but the cere is covered more with cartilaginous papillae, the

casque is taller but more withered basally and strongly backward curving; the mantle, body and wing spots and vermiculations, are larger and denser (Crowe *et al.* 1986).

N. m. coronata (Figure 1.1H) (including former *transvaalensis* and *limpopoensis*) occurs in the moister eastern parts of South Africa. It has been introduced into the Western Cape Province. It is different from *mitrata* in that the casque is much better developed and the mantle more streaked than barred with white (Crowe *et al.* 1986).

General Background

The wing structure and style of flight of guineafowl are characteristic of non-migratory galliform birds. The wings are rather small and rounded, designed for short bursts of rapid flight, enabling the birds to make a quick escape when danger threatens and are quite unsuitable for any form of sustained flight. Their style of flight involves an explosive take off and a series of rapid, powerful wing beats, then gliding down into cover (Crowe *et al.* 1986). In line with the limited flying abilities of the guineafowl is their short tail, with the Vulturine Guineafowl being an exception having a relatively long tail, particularly the central tail feathers (Hastings Belshaw 1985). The legs and feet are adapted to a terrestrial lifestyle with three front toes, a single hind one and strong claws (Ayeni 1983).

These features indicate that guineafowl mostly move about by walking. All guineafowl species have highly terrestrial lifestyles and fly only infrequently, normally either up onto their roosts at night, or in order to escape from predators, although in the latter case they often prefer to run off at great speed (Crowe *et al.* 1986). They are almost exclusively ground foragers and tend to spend the greater part of the day roaming about their territories, using their feet and bill to excavate food items (Hastings Belshaw 1985). All species of guineafowl are sedentary, and their movements are limited to the localised ones that they perform daily, over variable circuits, in search of food and water (Crowe 1978; Del Hoyo *et al.* 1994). Despite their apparent reluctance to fly, guineafowl are capable of high mobility, and can cover as much as 30 to 50 kilometres during the day walking from place to place while foraging (Hastings Belshaw 1985).

They are gregarious for most of the year when not breeding, but at the onset of the breeding season, the flocks disperse, so that only solitary individuals or monogamous pairs are seen, or the odd small group perhaps composed of non-breeders (Crowe *et al.* 1986). The flocks are governed by a complex social structure, and research on the Helmeted Guineafowl has shown that, at least in this species, individuals can remain in the same flock over a period of several years (Crowe 2000). Group size can vary quite considerably from species to species, with the Black Guineafowl normally forming groups of fewer than ten individuals and the Helmeted Guineafowl capable of

gathering in flocks of over 200 birds, although normally this species forms smaller flocks of 15-40 birds (Little 1997). The other species form flocks of intermediate size (Del Hoyo *et al.* 1994).

There is a paucity of information on the breeding habits of most of the members in the family, with the colour and size of the eggs constituting all available knowledge in some species. Thus all details concerning courtship display, the roles of the sexes, the incubation period, overall levels of success, and so on, can only be inferred tentatively from the best known species, the Helmeted Guineafowl (Crowe *et al.* 1986). In all species, sexual dimorphism is limited merely to males being slightly larger than females with no evident differences in plumage. There is no seasonal variation in plumage in any of the species (Del Hoyo *et al.* 1994). The Helmeted, Crested and Vulturine Guineafowl are monogamous, although males sometimes attempt to mate with other, unattended females (Elbin *et al.* 1986). The Plumed Guineafowl is probably monogamous too, while individual males of the Black Guineafowl have been seen with two females (Del Hoyo *et al.* 1994).

Clutch size is normally 4-19 eggs, but even larger clutches have been recorded, although these probably refer to cases of more than one female laying in the same nest. There is no record of any species ever laying more than a single clutch per season (Hastings Belshaw 1985). Once the breeding season draws to a close, the family group is merged into a much larger flock, consisting of other similar family groups and some non-breeding birds. Within this larger flock, the unity of the family group tends to persist, and the young birds remain in the company of their parents for at least a further two or three months. Although flocks are fairly stable, individuals do not necessarily return to the same flock that they belonged to before the breeding season (Del Hoyo *et al.* 1994).

A limiting factor in the distribution of guineafowl is the proximity of water holes or other sources of drinking water (Wolff and Milstein 1987; Pero and Crowe 1996). They are rarely found further than 10km away from water, lakes and large rivers or smaller perennial ones (Hastings Belshaw 1985). The Vulturine Guineafowl is once again an exception, as this species has an unusually long caecum constituting over 23% of the entire length of the intestine, which may permit it to regulate and limit water loss (Del Hoyo *et al.* 1994). Another necessity, for all species, is the presence of suitable trees for roosting in at night. On occasions when there may not be suitable roosting trees in the vicinity, the Helmeted Guineafowl, at least, will roost on some sort of substitute, having been observed roosting on telephone poles (Little 1997).

Humans and guineafowl have a relationship that goes into ancient times. This relationship is mainly as a result of the domestication of the Helmeted Guineafowl. In all likelihood, several different peoples have domesticated this species independently at different times (Hastings Belshaw 1985). There are references to guineafowl as early as 2400 BC on murals in the fifth dynasty Egyptian pyramid of Wenis at Saqqara. The Phoenicians and Greeks, the latter at least as

early as 400 BC, kept domesticated guineafowl. Later in the fourth century BC, the Moroccan race *sabyi* of the Helmeted Guineafowl was considered a sacred bird on one of the islands of the Aegean. The Romans regularly kept birds of both this (*sabyi*) and the nominate race (*meleagris*) for food, which they moved to all parts of their vast empire. With the fall of the Roman Empire, however, the species disappeared from Europe, leaving a long period in which it does not feature in historical records, apart from an odd record of some captive birds in Athens in the tenth century AD (Hastings Belshaw 1985). Portuguese explorers and navigators subsequently brought back individuals of the West African race *galeata* to Europe in the fifteenth and sixteenth centuries, and the species was regularly kept once again. Due to its repeated domestication over many centuries this species is present practically all over the world, usually in domestic form, but in many cases with feral populations too. These feral populations arose where a proportion of the domestic stock escaped and managed to establish more or less stable populations in the foreign habitat at the same time hybridizing with indigenous populations (Del Hoyo *et al.* 1994).

Molecular systematics and phylogeography

The avian mitochondrial DNA (mtDNA) genome provides a useful tool for investigating evolutionary relationships both within and between species (Shields and Helm-Bychowski 1988; Quinn 1997). Mitochondrial DNA seemingly has enormous value for resolving the phylogenies of recently evolved avian taxa, and numerous phylogenetic studies of avian groups have been carried out using mtDNA variation as a source of characters (Kimball *et al.* 1999; Fuchs *et al.* 2004; Lijtmaer *et al.* 2004). Mitochondrial DNA is attractive for phylogenetic studies because of its conservative evolution with regard to gene order and, in its protein-coding genes, conservative amino acid replacement and occurrence of insertions and deletions contrasted with a high rate of synonymous substitutions. Two different regions of the mitochondrial genome, cytochrome *b* (cyt *b*) and the control region (CR), were used in the phylogenetic analyses of this study.

The mitochondrially encoded cytochrome *b* gene has been used most often in avian phylogenetic studies based on DNA sequences and has proved a useful tool for investigating evolutionary relationships both within and between species (Voelker 2002; Thomassen *et al.* 2003; Moyle 2004; Barker 2004). The control region of the mtDNA genome has successfully been used in identifying relationships among morphologically recognised subspecies (Baker and Marshall 1997; Questiau *et al.* 1998; Zink *et al.* 2003; Barrowclough *et al.* 2004; Burg and Croxall 2004). It has also proved very efficient in resolving phylogenetic relationships at much deeper levels such as the family level (Douzery and Randi 1997; Saunders and Edwards 2000; Donne-Goussé *et al.* 2002).

With the advent of modern molecular studies, geographically based DNA analysis below the species level, termed phylogeography (Avise *et al.* 1987), maintains a prominent role in

evolutionary biology since it provides a window on the role of gene flow in the process of speciation. Phylogeographic structure of any species reflects patterns of historical fragmentation and processes such as restricted gene flow, selection, mutation and drift, and species-specific dispersal capabilities (Slatkin 1987; Avise *et al.* 1989; Avise 1994 and references therein). The distribution of genetic variability within and among populations is affected both by recurrent factors and historical events. Historical factors are generally expected to be more influential in taxa with comparatively low vagility (Phillips 1994), a common outcome being the detection of strongly defined associations between mtDNA haplotypes and geographical location. Using a fast-evolving marker such as the control region in animal mtDNA, population genetics and phylogenetic methods can be combined to infer species recent history and evolution.

The control region is characterised by rapid change in sequence and length (Saccone *et al.* 1991) and is therefore well suited to population genetic analysis. Several authors (Wenink *et al.* 1994; Baker and Marshall 1997; Piertney *et al.* 2000; Girman *et al.* 2001; Randi *et al.* 2003; Gay *et al.* 2004) have identified the control region as being a useful tool in assessing the extent of gene flow between populations.

Many bird species are subdivided into several subspecies based on morphological data (Questiau *et al.* 1998). In intraspecific phylogeography studies using mtDNA, these polytypic species can be classified into several categories corresponding to the models defined by Avise *et al.* (1987). The two main categories can be described as follows: (i) strong phylogeographic structure of mitochondrial haplotypes in agreement with the distribution of the previous described morphs that are biogeographically isolated, such as the bluethroat *Luscinia svecica* (Questiau *et al.* 1998), the rock partridge *Alectoris graeca* (Randi *et al.* 2003), the blue grouse *Dendragapus obscurus* (Barrowclough *et al.* 2004), the loggerhead shrike *Lanius ludovicianus* (Eggert *et al.* 2004), and the bush-tanager *Chlorospingus ophthalmicus* (Garcia-Moreno *et al.* 2004); and (ii) genetic homogeneity without any biogeographic structure even if the species is morphologically and geographically polytypic such as the redpoll finch (Seutin *et al.* 1995), the great spotted woodpecker (Zink *et al.* 2002), and the yellow wagtail (Pavlova *et al.* 2003).

The mitochondrial genome in birds is, however, maternally inherited without recombination, and therefore, analysis of mtDNA polymorphism yields a female-biased description of the population structure. Analysis of nuclear markers, such as microsatellites or nuclear DNA introns, would provide a necessary complement to the mtDNA data, giving a more complete and accurate picture of the population structure.

Reliable estimates of subspecific and population differentiation are crucial in conservation biology, where it is necessary to understand whether populations are genetically isolated from each other,

and if so, to what extent (Smith *et al.* 2000). The knowledge of population structuring may therefore provide valuable guidelines for conservation strategies and management (Erwin 1991).

Aims

The aims of the present study were to:

1. Determine whether maternal mtDNA molecular data described concordant subspecies divisions and to what degree the subspecies boundaries are consistent with mtDNA gene trees.

2. Assess the mtDNA variability within and among populations of Helmeted Guineafowl, and subsequently to draw inferences on demographic processes and determine the degree of geographical genetic structuring.

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Chapter 2

Molecular differentiation among the subspecies of Helmeted Guineafowl (*Numida meleagris*)

Molecular differentiation among the subspecies of Helmeted Guineafowl (*Numida meleagris*)

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Abstract

There are nine recognised subspecies of Helmeted Guineafowl (*Numida meleagris*) in Africa. The subspecific delineation is based on geographical morphological character variation. Two different regions of the mitochondrial genome were used in this study to determine the molecular phylogenetic relationships among six of the subspecies. Cytochrome *b* and control region sequences were used as well as the combined sequences of the two. The concordance of the mtDNA molecular data and current subspecific designations was then examined. Phylogenetic analysis of the mtDNA sequences resulted in the identification of four clades. There was a very distinct southern African clade, and to a lesser extent northern, eastern and West African clades. Although the genetic structuring apparent among the Helmeted Guineafowl populations showed some geographic consistency, it did not concur strongly with the previously recognised subspecies boundaries. This genetic structuring was hypothesised to have been driven by the cyclical expansion and contraction of the *Brachystegia* woodland caused by climatic fluctuations during the Pleistocene.

Keywords: Helmeted Guineafowl, *Numida meleagris*, mtDNA, cytochrome *b*, control region, molecular systematics, *Brachystegia* woodland.

Introduction

The Helmeted Guineafowl, *Numida meleagris* (Linnaeus, 1758), is Africa's most widespread terrestrial gamebird (Crowe *et al.* 1986) (Figure 2.1). It is locally common to abundant in virtually all open-country terrain, from forest edge to subdesert steppe and the bases of mountains (Crowe *et al.* 1986; 2000). Resident from Senegal to Ethiopia and Somalia, south to Namibia and South Africa (Little and Crowe 2000). It is most common in wooded moist grass savannas with 400-1200 mm rainfall, with especially high concentrations in savannas mixed with cultivated wheat and maize. Critical habitat features are the availability of drinking water, cover and elevated nightly roosting sites (Crowe *et al.* 1986; 2000).

Over 30 subspecies have been recognised within the single species *N. meleagris* based on geographical morphological character variation. The main morphological characters used for classification were the variation in shape, size and colour of the face, helmet and wattles, as well as the degree to which they possess wart or tuft-like growths around and above their nares. Over the years, anywhere from 14 to 24 subspecies were recognised at one time (Chapin 1932; Peters 1934; Ghigi 1936; Mackworth-Praed and Grant 1952; Boetticher 1954; Mackworth-Praed and Grant 1962; White 1965; Mackworth-Praed and Grant 1970) (see Table 1 in Crowe 1978 for a summary of the taxonomy of guineafowl). The taxonomy and morphology of the Helmeted Guineafowl were most recently reviewed by Crowe (1978), who determined that nine subspecies warranted recognition. Crowe (1978) followed the taxo-evolutionary subspecies concept of Ford (1974), whose concept limits the awarding of subspecies status to geographic aggregates of populations which appear to have undergone genetic and phenotypic divergence in allopatry.

Six of the nine subspecies are represented in this study (Figure 2.1), namely: *N. m. galeata* (West Africa and northern Angola), *N. m. meleagris* (Chad to Ethiopia, Uganda and northern Kenya), *N. m. mitrata, N. m. coronata* (South Africa), *N. m. damarensis* (southern Angola to Botswana and Namibia) and *N. m. reichenowi* (Kenya and central Tanzania). The three subspecies not sampled are *N. m. somaliensis, N. m. marungensis* and *N. m. sabyi*; the latter is probably now extinct. All of these subspecies are morphologically distinct and occur in discrete geographical areas, but do form zones of intergradation with at least one other subspecies (Crowe 1978; Crowe and Snow 1978).

It seems unlikely that this sometimes striking, effectively qualitative, geographical variation in head colour and adornments among various subspecies has any adaptive value (Crowe 1979), plays any role in mate recognition or inhibits interbreeding between members of the various subspecies (Crowe 2000). Indeed, within the zones of natural geographic contact between subspecies of the

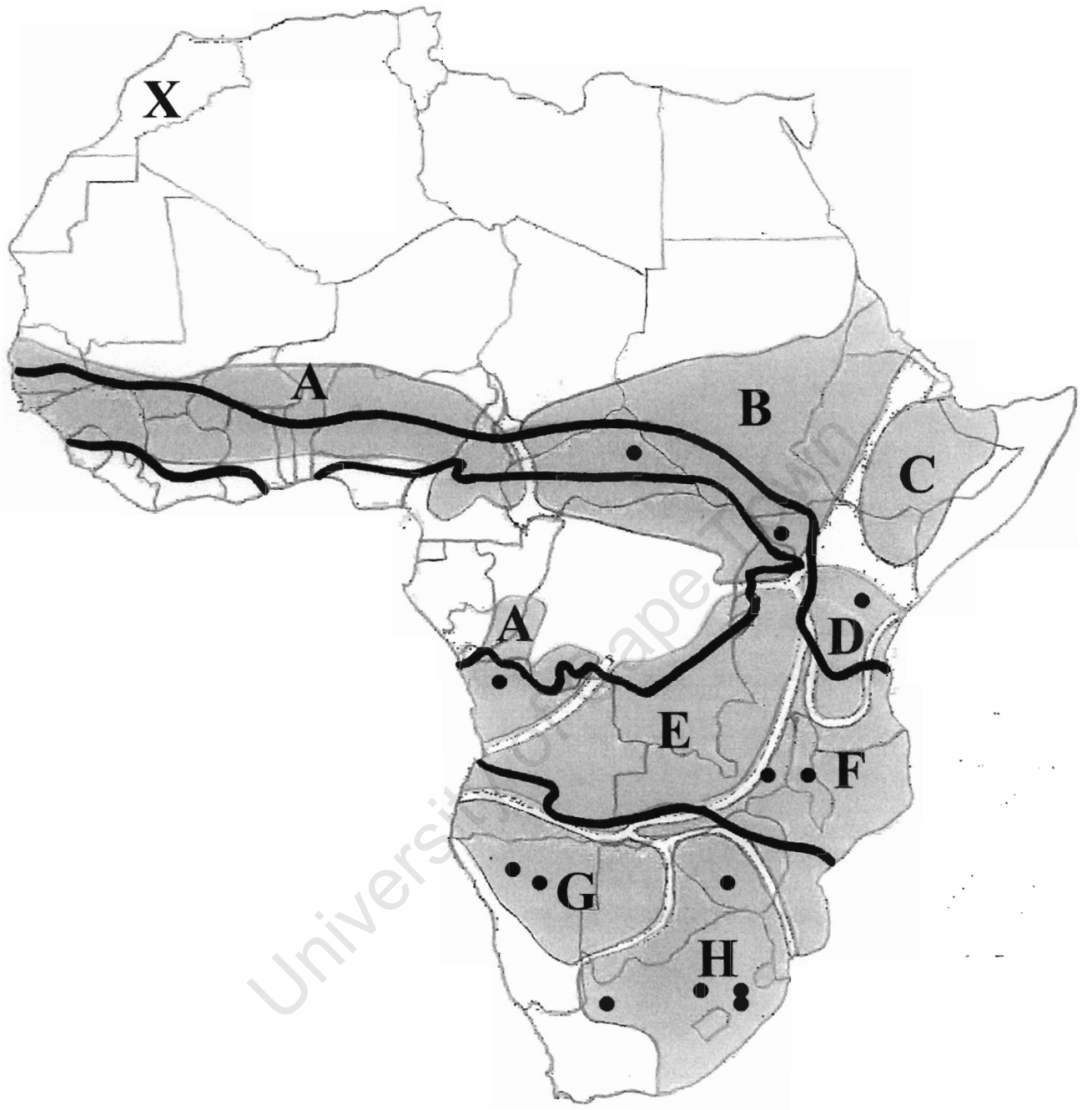

Figure 2.1. The distribution of nine subspecies of Helmeted Guineafowl (*Numida meleagris)* and the country where collected. **A** - *N. m. galeata* (Angola), **B** - *N. m. meleagris* (Central African Republic & Uganda), **C** - *N. m. somaliensis*, **D** - *N. m. reichenowi* (Kenya), **E** - *N. m. marungensis*, **F** - *N. m. mitrata* (Zambia & Malawi), **G** - *N. m. damarensis* (Namibia), **H** - *N. m. coronata* (South Africa), **X** – *N. m sabyi* (Modified from Crowe & Snow 1978) Intermediate forms are found in narrow hybrid zones between subspecies boundaries.

● - Study sites

— - Limits of *Brachystegia* woodland (from Coe and Skinner 1993)

Helmeted Guineafowl, there is apparently free interbreeding, producing anatomically intermediate forms of all persuasion (Crowe 1978).

To understand the genetic relationship underlying the morphologically different subspecies described, molecular data were generated. The avian mitochondrial DNA (mtDNA) genome provides a useful tool for investigating evolutionary relationships both within and between species (Shields and Helm-Bychowski 1988; Quinn 1997). Two different regions of the mitochondrial genome, cytochrome *b* (cyt *b*) and the control region (CR), were used in this study due to different constraints acting on each of these regions.

The CR has conserved functional motifs (Sbisa 1997; Randi and Lucchini 1998) but accumulates mutations three to five times faster than cyt *b* which is a protein coding gene (Meyer 1994). It has, however, been found that in gnatcatchers the CR is no more variable than cyt *b* (Zink and Blackwell 1998). The control region has successfully been used in determining population structure within species and for identifying relationships among morphologically recognised subspecies (Baker and Marshall 1997; Questiau *et al.* 1998; Zink *et al.* 2003; Barrowclough *et al.* 2004; Burg and Croxall 2004). Indeed, it has even proven to be an efficient tool with which to decipher phylogenies not only at the species and genus levels, but also at the family level (Kimball *et al.* 1999; Lucchini and Randi 1999; Donne-Goussé *et al.* 2002). The cyt *b* gene has been used most often in avian phylogenetic studies based on DNA sequences and has proved a useful tool for investigating evolutionary relationships both within and between species (Voelker 2002; Thomassen *et al.* 2003; Barker 2004; Moyle 2004).

In this paper we examined the mtDNA cyt *b* and control region variation among populations of six subspecies of Helmeted Guineafowl. The aim of the present study was to determine whether this variation is congruent with the morphological variation used to delineate subspecies.

Materials and Methods

Study area and sampling

Samples of the six subspecies were collected from across Africa (Figure. 2.1) in the form of blood or tissue. The localities and numbers of individuals collected for each subspecies are presented in Table 2.1. In addition to the sampling of subspecies across Africa there was also sampling throughout South Africa, where the introduction of domesticated guineafowl has led to introgression with natural populations (Rossouw 1996; Walker 2000; Walker *et al.* in press).

Table 2.1 Subspecies of Helmeted Guineafowl and the localities of populations sampled

Subspecies	Locality (no. of individuals)
N. m. galeata	North-west Angola (1)
N. m. meleagris	Central African Republic (2) Uganda (2)
N. m. mitrata	Zambia (1) Malawi (2)
N. m. coronata	Zimbabwe (1) South Africa (4)
N. m. damarensis	Namibia (2)
N. m. reichenowi	Kenya (2)

Sequences from the Central African Republic and one of the samples from Kenya were obtained from Rossouw (1996). The outgroup sequence of *Acryllium vulturinum*, as well as sequences of *Guttera pucherani* were also obtained from Rossouw (1996).

DNA extraction, PCR amplification and sequencing

DNA was extracted from all blood and tissue samples using a standard Proteinase K digestion followed by phenol/chloroform extraction (Sambrook *et al.* 1989). The digestion was performed in 500µl of extraction buffer (0.05M Tris-HCl, 0.001M EDTA·Na_2, 0.1M NaCl, 0.5%SDS) with 50µl Proteinase K (10µg/ml) (Roche Diagnostics). Samples were digested overnight at 55˚C followed by incubation for 1 hour at 37˚C with 60µl RNase A (1 mg/ml) (Roche Diagnostics). Thereafter samples were extracted twice with phenol and once with a chloroform:isoamyl alcohol (24:1) solution. Samples were then precipitated overnight at -20˚C in a solution containing 0.1 volumes 3M Sodium Acetate and 2 volumes of 96% Ethanol. The genomic DNA was finally pelleted in a desktop microcentrifuge at 14000rpm and resuspended in 50µl Sabax® water (Adcock Ingram). The 5' end of cyt *b* and the 5' domain of the CR were amplified by polymerase chain reaction (PCR, Saiki *et al.* 1988). The primers L14841 (Kocher *et al.* 1989) and H15696 (Primer H15547 of Edwards *et al.* 1991) were used to amplify a 706 base pair part of the 5' end of cyt *b*. The 5' end of the CR, 550 base pairs, was amplified using primers L16747 (Wenink *et al.* 1994) and H522 (Quinn and Wilson 1993).

Approximately 100ng of genomic DNA was used as template in a total PCR reaction volume of 50µl. In addition to the genomic DNA, the reaction contained 2mM $MgCl_2$, 1 x reaction buffer, 0.2mM of each of the four nucleotides, 12.5 picamol of each primer and 1.5U of Super-therm® DNA polymerase (Southern Cross Biotechnology). A Geneamp® PCR System 9700 (Applied Biosystems) was used to cycle the reaction mix through the following conditions: denaturing at 94°C for 2min followed by 35 cycles of denaturing at 94°C for 30 seconds, primer annealing at 52-56°C for 30 seconds and elongation at 72°C for 30 seconds; and finally an extended elongation period of 10 minutes at 72°C. Precipitating with an Ethanol and Sodium Acetate solution purified the PCR products. Dye-terminator (Sanger *et al.* 1977) cycle sequencing was performed, using primers L14841 and H15696 for cyt *b*, and L16747 and H522 for the CR, with the BigDye DNA Ready Reaction sequencing kit (Applied Biosystems) in a Geneamp® PCR System 9700 (Applied Biosystems). Sequencing was performed in quarter reactions in 10µl according to the manufacturer's instructions. The cycle sequencing products were purified by precipitating with an Ethanol and Sodium Acetate solution. Thereafter, nucleotide sequences were determined through electrophoresis on an ABI 3100 automated sequencer (Applied Biosystems).

Sequence analysis

Nuclear copies of mitochondrial genes (called numts) have been documented in a wide variety of organisms (Sorenson and Fleischer 1996; Zhang and Hewitt 1996; Quinn 1997; Sorenson and Quinn 1998) and there is a widespread concern over their effects on studies of molecular systematics and population biology. We are confident that all of the sequences obtained in this study are mitochondrial in origin. Almost all sample DNA was extracted from tissue and not blood, as blood is known to be prone to amplification of numts since it is poor in mtDNA (Quinn 1992; Arctander 1995; Sorenson and Fleischer 1996). Additionally we detected no evidence of multiple copies of the CR or cyt *b* in any of our sequences suggesting that our primers had not amplified a mixture of mitochondrial and nuclear copies.

Heavy and light strand sequences for both cyt *b* and CR were imported into Sequence Navigator version 1.0.1 (Applied Biosystems) where they were proofread for each sample. Consensus sequences of each sample were thereafter aligned using CLUSTAL X version 1.74 (Thompson *et al.* 1997). Phylogenetic trees were reconstructed in PAUP* version 4.0b10 (Swofford 2000) using: 1) the neighbour-joining algorithm (Saitou and Nei 1987) with pairwise HKY85 corrected distances (Hasegawa *et al.* 1985); 2) parsimony (Kluge and Farris 1969; Farris *et al.* 1970), using the heuristic search criterion with 1000 random addition sequence replicates, multiple minimal trees swapped by tree bisection and reconnection, and collapsed zero length branches. A strict consensus tree was constructed from multiple equally parsimonious trees. Statistical support of the consensus topology of the parsimony tree and the neighbour-joining tree were assessed by bootstrapping (Felsenstein 1985) with 1 000 replicates. The parameters for the neighbour-joining

analysis were selected by hierarchical likelihood ratio testing using MODELTEST 3.06 (Posada and Crandall 1998), the AIC criteria was followed. To determine whether the two data sets could be combined a partition homogeneity test was implemented as in PAUP* version 4.0b10 using 1000 replicates (Farris *et al.* 1995). Although this test is controversial (Yoder *et al.* 2001 and Hipp *et al.* 2004), combining the two data sets is justified by the two regions being on a single locus (mtDNA) and no conflicting nodes obtained that were strongly supported by bootstrap. Phylogenetic trees were rooted using homologous CR and cyt *b* sequences of *Acryllium vulturinum*.

Results

The variability of the two regions was extremely contrasting, with the CR exhibiting the most variability of the two with 14.3% of all sites variable and cyt *b* only having 1.9% of sites variable. The best-fit model for the data, calculated by Modeltest (Posada and Crandall 1998), was the HKY85 model of substitution (Hasegawa *et al.* 1985). The pairwise estimates of nucleotide sequence divergence (%HKY85) for both the control region and cytochrome *b* are represented in Table 2.2. Overall the divergences ranged between 0.00 and 1.39% for cyt *b* and between 0.00 and 9.18 % for CR.

Control region

There were 15 unique haplotypes from the 18 individuals sampled. Of the 322 characters, 56 were parsimony informative yielding one most parsimonious tree (CI = 0.63, HI = 0.37, RI = 0.79). The control region sequences for 18 Helmeted Guineafowl individuals sampled throughout Africa provided almost identical topologies for both neighbour-joining and parsimony trees (Figures 2.2a and 2.2b). Bootstrap support for both trees was very similar, identifying at least two main clades within *N. meleagris* and at most four clades.

The first main clade contained sequences from northern, eastern and West Africa, indicated by their respectively numbered clades 1, 2 and 3 in Figure 2b. Uganda and the Central African Republic (Clade 1) represent the northern distribution of Helmeted Guineafowl and the subspecies *N. m. meleagris* (Figure 2.1). Included in this clade was an individual from Kenya (*N. m. reichenowi*), which has a more eastern distribution. There was strong support for this group in the phylogram (Figure 2.2a) with a bootstrap value of 99%, with the cladogram (Figure 2.2b) exhibiting more moderate support with a 70% bootstrap value. The eastern clade (Clade 2) consisted of samples from Kenya (*N. m. reichenowi*), Malawi (*N. m. mitrata*) and Zambia (*N. m. mitrata*) with one sequence from South Africa (*N. m. coronata*). Clade 2 also exhibited strong support using both distance and parsimony analyses with bootstrap values of 100% and 88% respectively. The

third clade displayed a West African distribution with two samples representing the subspecies *N. m. galeata* (one from a wild population in Angola and the other sampled from domesticated stock in Europe). Also in this third clade was an individual *N. m. mitrata* sample from Malawi. The nodal support for clade 3 was also strong with 98% and 88% bootstrap values. The eastern and West African clades (Clades 2 and 3) formed a group that had fairly weak support as shown in Figures 2.2a and 2.2b with bootstrap values of 62% and 69%. The overall group containing clades 1, 2 and 3 was weakly supported with 64% for the distance analysis and 57% using parsimony.

The second main clade comprised only sequences from southern Africa with very strong neighbour-joining bootstrap support of 99% (Figure 2.2a) and a strong parsimony bootstrap value of 86% (Figure 2.2b). The guineafowl of this southern African clade belong to the subspecies *N. m. coronata* and *N. m. damarensis* with distributions in South Africa and Zimbabwe, and Namibia respectively.

Highlighted in Table 2.2 are the sequence divergences of the control region for the four clades identified in Figure 2.2a. HKY85 sequence divergences ranged from 0% to 3.56% for haplotypes within each clade and from 4.37% to 9.15% between clades (mean = 6.79%, SD = 1.11%). The highlighted blocks represent sequentially, down the diagonal, the northern clade, the eastern clade, the West African clade and the southern clade.

Cytochrome b

Cytochrome *b* sequences of 13 individuals of Helmeted Guineafowl, representative of the four clades exhibited in the control region analysis, were also analysed using neighbour-joining and parsimony methods (Figures 2.3a and 2.3b). A strict consensus tree was constructed from 89 equally parsimonious trees (CI = 0.87, HI = 0.13, RI = 0.69). There were 32 parsimony informative characters in the data set of 589 characters. Of the 13 individuals sampled, there were eight unique haplotypes. The first clade from the control region analysis (the northern clade) was also resolved in the cytochrome *b* analysis, with bootstrap support of 78% in the phylogram (Figure 2.3a) and 76% in the cladogram (Figure 2.3b) for the monophyly of the Kenyan and Central African Republic samples. There was no support for any of the other clades (southern/eastern/western) although the topology of the phylogram (Figure 2.3a) indicated a slight divergence from the northern clade. The West African clade, represented by the domesticated *N. m. galeata* and the Malawian *N. m. mitrata* had some support (51% bootstrap) in the phylogram (Figure 2.3a) but none in the cladogram (Figure 2.3b). The eastern clade, Zambia (*N. m. mitrata*) and Kenya (*N. m. reichenowi*), was only represented by a single cytochrome b haplotype and its placement was unresolved. A similar pattern was shown in the southern African group, where divergence is evident from the phylogram (Figure 2.3a) but there was no bootstrap support for the clade.

Combined analysis

The test for partition homogeneity did not reject the congruence of the different data sets ($P < 0.05$). A combined analysis of both the control region and cytochrome *b* sequences resulted in one most parsimonious tree from 911 characters, of which 80 were parsimony informative. The most parsimonious tree is presented in Figure 2.4 (CI = 0.79, HI = 0.21, RI = 0.76). All four clades described for the control region sequences (northern, eastern, western and southern) were also present in the combined sequence analysis with bootstrap support of 94% for the northern clade, 89% for the eastern clade, 89% for the West African clade and 84% for the southern clade. The eastern and West African clades again grouped together, as in the control region analysis, with 72% bootstrap support, but in this analysis they were joined with reasonable confidence (68% bootstrap) to the northern clade. The southern clade still showed strong support as a monophyletic group, with 84% support.

Table 2.2. Pairwise estimates of nucleotide sequence divergence (% HKY85) for the control region below the diagonal and for cytochrome *b* above the diagonal for six subspecies of Helmeted Guineafowl

	N. m. meleagris (Uganda)	*N. m. meleagris* (Uganda)	*N. m. meleagris* (CAR)	*N. m. meleagris* (CAR)	*N. m. reichenowi* (Kenya)	*N. m. reichenowi* (Kenya)	*N. m. mitrata* (Malawi)	*N. m. coronata* (SA)	*N. m. mitrata* (Zambia)	*N. m. galeata* (Harrods)	*N. m. galeata* (Angola)	*N. m. mitrata* (Malawi)	*N. m. coronata* (SA)	*N. m. coronata* (SA)	*N. m. coronata* (Zimbabwe)	*N. m. damarensis* (Namibia)	*N. m. coronata* (SA)	*N. m. damarensis* (Namibia)	*Guttera pucherani*	*Guttera pucherani*	*Acryllium vulturinum*
N. m. meleagris (Uganda)																					
N. m. meleagris (Uganda)	0.00																				
N. m. meleagris (CAR)	1.92	1.92		0.00	0.51	0.86	1.20		0.86	1.03		1.21	1.39	1.39	1.21	0.99	1.21	1.21	11.08		11.06
N. m. meleagris (CAR)	1.91	1.91	0.00																		
N. m. reichenowi (Kenya)	2.23	2.23	2.91	2.89		0.68	0.00		0.68	0.51		0.68	1.38	1.38	1.03	0.73	1.03	1.03	11.00		10.55
N. m. reichenowi (Kenya)	5.24	5.24	6.32	6.65	5.90		0.34		0.00	0.17		0.34	0.68	0.68	0.34	0.24	0.34	0.34	10.78		10.54
N. m. mitrata (Malawi)	6.29	6.29	7.40	7.73	6.65	1.58															
N. m. coronata (SA)	6.29	6.29	7.41	7.74	6.65	1.58	0.63		0.00	0.17		0.34	0.68	0.68	0.34	0.24	0.34	0.34	10.78		10.54
N. m. mitrata (Zambia)	5.94	5.94	7.04	7.37	5.90	1.24	0.31	0.31		0.17		0.34	0.68	0.68	0.34	0.24	0.34	0.34	10.78		10.54
N. m. galeata (Harrods)	5.60	5.60	5.27	5.60	5.96	4.57	4.91	4.92	4.57			0.17	0.86	0.86	0.51	0.49	0.51	0.51	10.57		10.34
N. m. galeata (Angola)	5.59	5.59	5.98	6.30	6.66	5.26	4.91	4.92	4.57	1.91											
N. m. mitrata (Malawi)	5.59	5.59	5.98	6.30	6.25	4.99	4.68	4.68	4.37	1.88	0.00		1.03	1.03	0.68	0.49	0.68	0.68	10.78		10.54
N. m. coronata (SA)	6.62	6.62	7.00	7.34	7.70	6.62	6.27	6.27	5.92	7.38	6.66	6.66		0.00	0.34	0.71	0.34	0.34	10.78		10.54
N. m. coronata (SA)	8.04	8.04	8.44	8.78	9.15	8.04	6.96	6.96	6.60	8.82	7.35	7.35	1.26		0.34	0.71	0.34	0.34	10.78		10.54
N. m. coronata (Zimbabwe)	7.70	7.70	8.84	9.18	7.48	6.53	5.59	5.59	5.28	8.12	8.09	8.09	3.56	2.88		0.24	0.00	0.00	10.78		10.13
N. m. damarensis (Namibia)	6.98	6.98	8.10	8.44	7.34	6.98	6.62	6.62	6.27	8.09	8.09	8.09	2.23	2.23	1.26		0.24	0.24	12.67		12.08
N. m. coronata (SA)	7.70	7.70	8.10	8.44	7.34	7.70	7.34	7.34	6.98	7.37	7.36	7.36	2.89	2.88	1.91	0.63		0.00	10.78		10.13
N. m. damarensis (Namibia)	6.27	6.27	7.37	7.70	6.23	6.53	6.21	6.21	5.90	6.87	8.09	8.09	2.89	2.88	1.26	0.63	1.26		10.78		10.13
Guttera pucherani	14.74	14.74	13.62	13.56	14.33	15.17	15.17	15.18	14.77	15.77	15.77	15.77	13.24	12.81	12.82	13.62	13.63	12.82			11.67
Guttera pucherani	14.68	14.68	13.55	13.50	12.70	14.70	13.90	13.91	13.51	13.62	13.63	13.63	12.78	12.37	11.62	13.17	12.39	12.39	6.68		
Acryllium vulturinum	14.73	14.73	15.62	15.54	12.86	14.80	14.80	14.79	14.48	13.91	16.61	16.61	16.94	17.35	16.08	16.92	16.05	16.07	12.55	11.72	

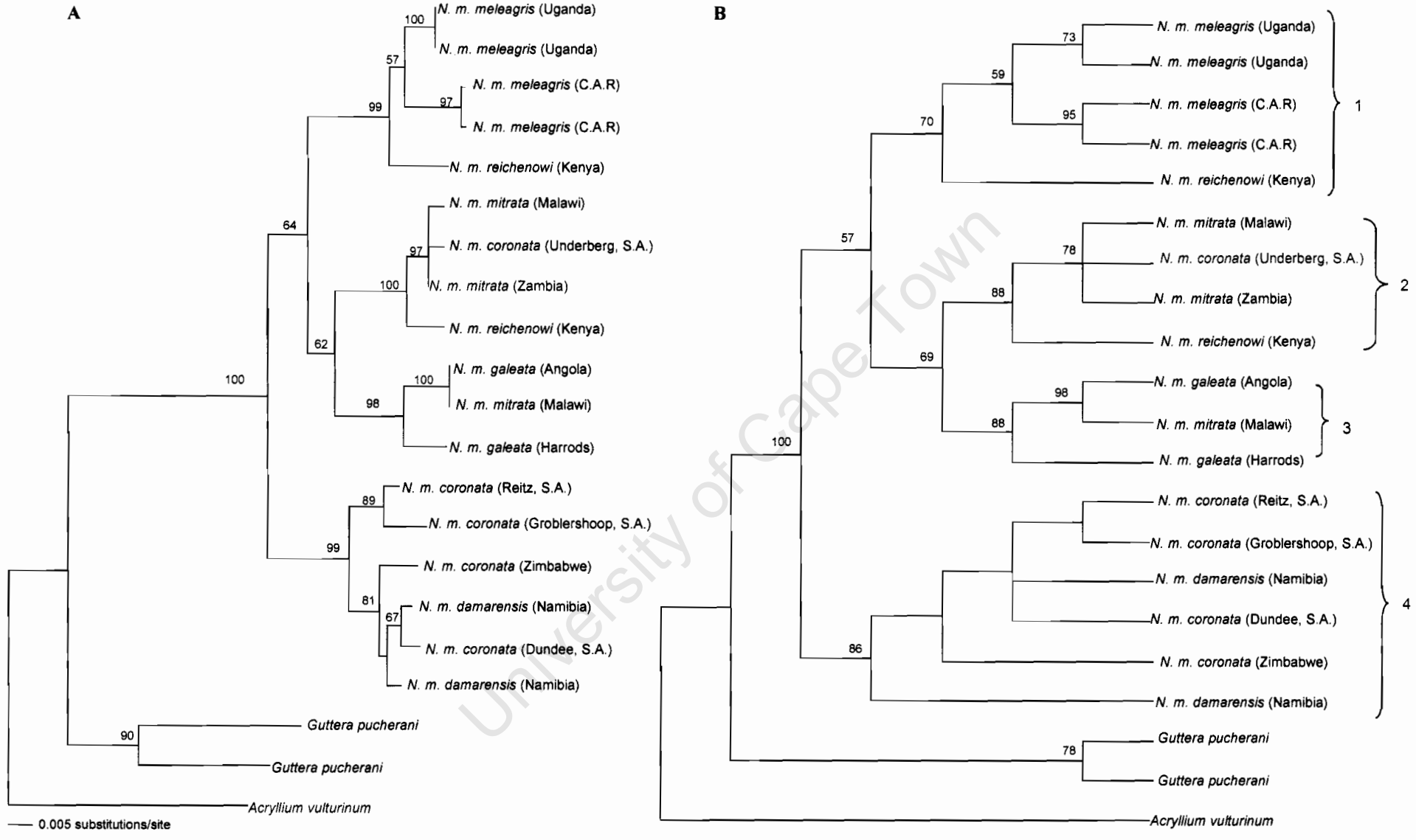

Figure 2.2. Phylogenetic reconstruction of the relationships among the six subspecies of guineafowl from control region sequences. (A) Distance analysis calculated with the neighbour-joining method using HKY85 corrected distances, 1000 bootstrap replicates were calculated. (B) Parsimony analysis using a heuristic search with 1000 bootstrap replicates. Only bootstrap values above 50% are indicated. [CAR – Central African Republic, S.A – South Africa, Harrods – a domesticated guineafowl used in comparative analysis with *N. m. galeata*] Numbered clades: 1 - northern clade, 2 - eastern clade, 3 - West African clade and 4 - southern clade

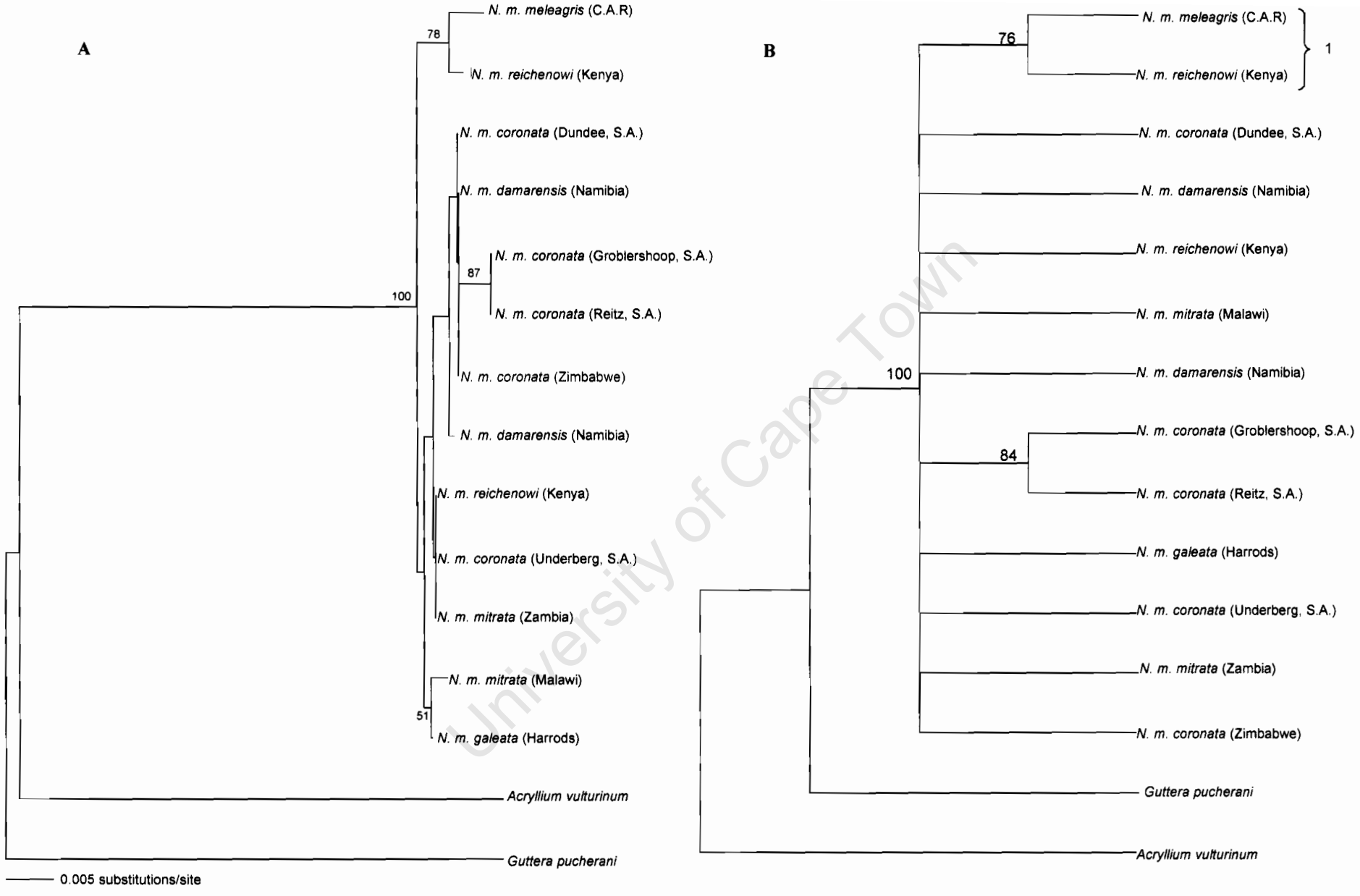

Figure 2.3. Phylogenetic reconstruction of the relationships among the six subspecies of guineafowl from cytochrome *b* sequences. (A) Distance analysis calculated with the neighbour-joining method using HKY85 corrected distances, 1000 bootstrap replicates were calculated. (B) Parsimony analysis using a heuristic search with 1000 bootstrap replicates. Only bootstrap values above 50% are indicated. [CAR – Central African Republic, S.A – South Africa, Harrods – a domesticated guineafowl used in comparative analysis with *N. m. galeata*]

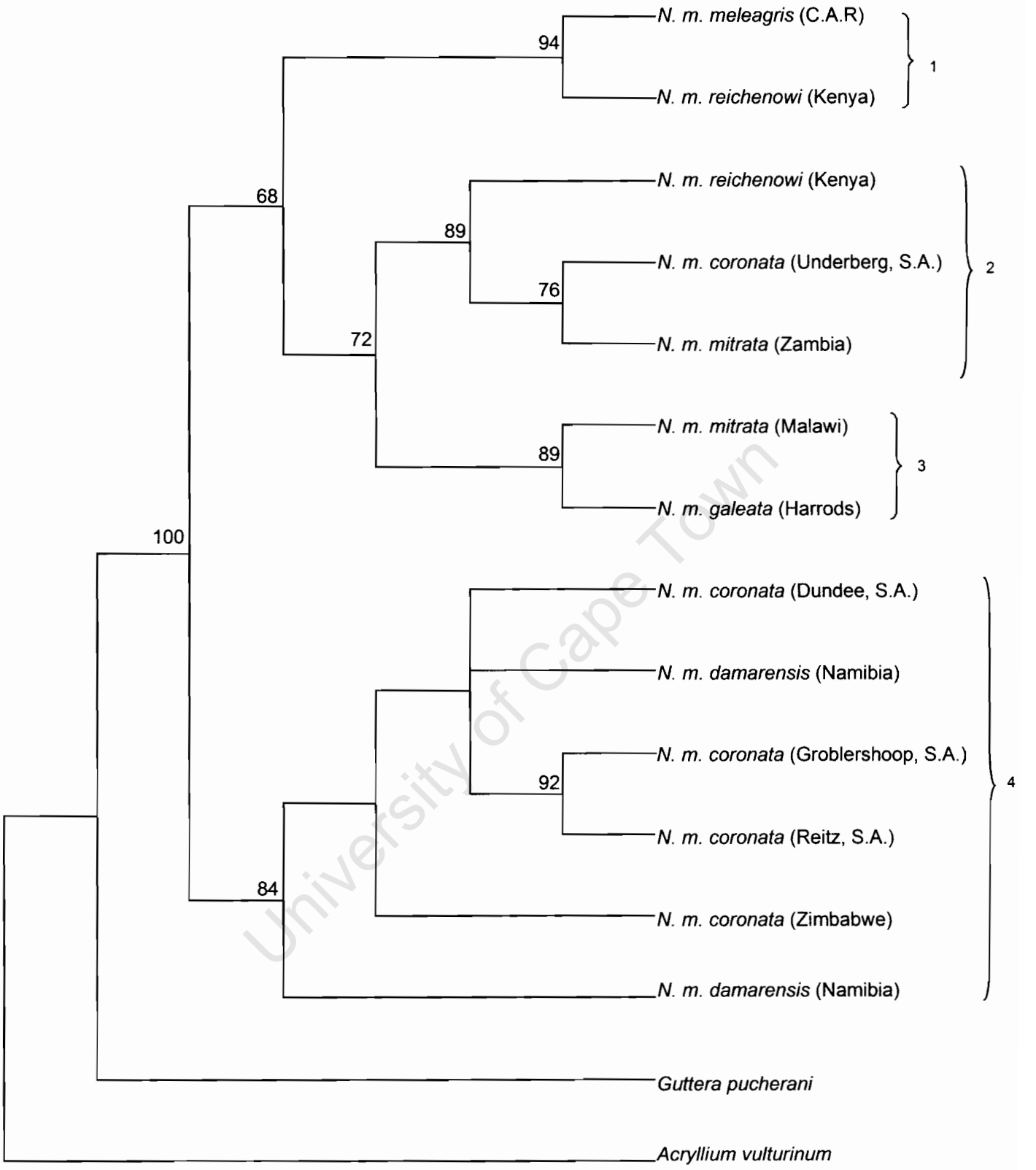

Figure 2.4. Phylogenetic reconstruction of the relationships among six subspecies of Helmeted Guineafowl (*Numida meleagris*) from combined analysis of control region and cytochrome *b* sequences. [CAR - Central African Republic, S.A. - South Africa, Harrods - a domesticated guineafowl used in comparative analysis with *N. m.* galeata] Numbered clades: 1 - northern clade, 2 - eastern clade, 3 - West African clade and 4 - southern clade.

Discussion

Helmeted Guineafowl have been domesticated since the time of ancient Rome and Greece, with the most recent domesticated stock originating from West Africa (*N. m. galeata*) (Ghigi 1936). These domesticated guineafowl have subsequently been introduced throughout the world and have been found to interbreed with wild populations forming morphological intermediates and in some cases there is introgression with little or no morphological differentiation (Rossouw 1996; Walker 2000; Walker *et al.* in press). We should therefore take care when looking at genetic variation among subspecies of Helmeted Guineafowl that we are not inadvertently sampling hybrids of domesticated birds that morphologically may resemble wild birds of that region. It is with this in mind that a domesticated guineafowl sample obtained from Harrods of London was included in this study. First, some morphologically indistinguishable domesticated birds in the wild would group closely with this sample known to be of domesticated origin (see *N. m. mitrata* from Malawi in Figure 2.4) and secondly, as the original stock of domesticated birds was from West Africa (*N. m. galeata*) this sample would represent the subspecies *N. m. galeata* in this study.

It was noted that a sample of *N. m. mitrata* from Malawi (eastern Africa) formed a clade (Figure 2.2b clade 3) with the domesticated bird and a sample from Angola, both representing the West African subspecies *N. m. galeata.* The collector of this particular bird noted that there were some morphological features similar to that of a domesticated bird (Bowie pers. comm.). This clade (Figure 2.2b clade 3) was therefore referred to as the West African clade (*N. m. galeata*) throughout this study, even though it included an individual purported to belong to the subspecies *N. m. mitrata* from the east, as it was evident from genetic analysis that this individual was originally from *N. m. galeata* stock. Inclusive of this West African clade there are four distinct clades of haplotypes (Figure 2.2b and Figure 2.4), each of which corresponds to a geographical area.

Environmental variation during the Pleistocene would have had an influence in shaping the evolution of the Helmeted Guineafowl within each area. In Africa such variation is related to a combination of climatic changes associated with glacial-interglacial periods and direct climate change (de Menocal 1995).

Although all four of the clades are distinct and well supported, the relationships that they have with one another is not as clear. The clades from eastern and West Africa (Clades 2 and 3) have strong support as being closely related to one another as evidenced by control region sequences (Figure 2.2b) and combined cytochrome *b* sequences with control region sequences (Figure 2.4). The relationship of the east/west clade with the northern and southern clades, however, is not completely unambiguous. Even though the control region sequence analyses (Figure 2.2) and

combined sequence analysis (Figure 2.4) place the east/west clade with the northern clade, the support for this grouping is only 68%. A population causing some confusion is the one sampled from Kenya (*N. m. reichenowi*) as one of the individuals falls within the northern clade and the other individual grouped within the eastern clade.

Although it may be premature to link any contributing factors to the patterns observed there are a few possible means by which these divergent clades could have arisen. First, the distribution of the *Brachystegia* ('miombo') woodland could have influenced the movements of the Helmeted Guineafowl (Figure 2.1). The *Brachystegia* woodland exhibited cycles of expansion and contraction throughout the Pleistocene (Hamilton 1976) with several species of birds and mammals having distributions interrupted by the expansion and contraction of the miombo woodland. Second, the rift valley has acted as a barrier to gene flow for bird species (Freitag and Robinson 1993), mammals (Matthee and Robinson 1997; Arctander *et al.* 1999) and insects (Lehmann *et al.* 2000). The African rainforest extends from the Gulf of Guinea to the western edge of the rift lakes. Conceivably, periods of rainforest expansion due to climate change may have been enough to decrease gene flow to a level that allowed for isolation and divergence of eastern and southern clades. This would act as a possible gene flow barrier as Helmeted Guineafowl only inhabit the forest edge and open habitat such as savannah and *Acacia* woodland. Thirdly, it has also been suggested that the development of grasslands in eastern Africa was relatively recent (0.6 Ma), although a period of grassland predominance was also detected between 1.7 and 1.2 Ma (Cerling 1992). This has led to the proposal that a number of mammal populations in eastern Africa were derived secondarily by migration from western, central-eastern and southern Africa after local extinctions in eastern Africa. These local extinctions could possibly be attributed to loss of habitat due to Pleistocene climatic changes (Arctander *et al.* 1999; Girman *et al.* 2001). Secondary migrations may then have occurred southwards from eastern Africa and northwards from southern Africa. This scenario would assume that there are no significant geographical barriers to dispersal between southern and eastern Africa, and between eastern and western Africa.

If the rift valley was the main barrier of gene flow between the Kenyan population and the northern and southern clades we would then expect to find large divergences between Kenya and the north, and Kenya and the south, as found in the ostrich (Freitag and Robinson 1993). This is not the case, as one of the Kenyan haplotypes groups with the northern clade and the other Kenyan haplotype forms part of the eastern clade suggesting that this might have been a recolonization event after a local extinction as hypothesised by Arctander *et al.* (1999). The proposed formation of an 'arid corridor' connecting the arid south-western and Saharo-Sindic regions of Africa at times of receding *Brachystegia* 'miombo' woodland (Verdcourt 1969; Kingdon 1971) would have allowed gene flow between eastern and southern Africa. Central Africa experienced a reduction in rainfall as recently as 11 000 to 12 000 years ago sufficiently severe to fragment what is now *Brachystegia*

woodland in Angola, Zambia, Zimbabwe, Mozambique and the south-eastern Congo and replace it with drier more open types of vegetation, such as *Acacia* wooded steppe (Moreau 1966; Hamilton 1976; 1982). This would have provided an opportunity for guineafowl in the west to move eastwards.

However, further analysis of Helmeted Guineafowl populations from central, western and eastern Africa is needed to evaluate this hypothesis more comprehensively. In particular, the inclusion of the subspecies *N. m. marungensis* might prove invaluable in determining gene flow as the distribution of *N. m. marungensis* is geographically central to the western, southern and eastern populations sampled in this study. Thorough sampling of individuals around the rift valley would allow us to see its impact on the Helmeted Guineafowl.

The southern clade is well supported and has a higher divergence within it than any of the other clades (2.04%), suggesting that this clade has been stable for a longer period of time than the others. This is supported by the fact that the climate of southern Africa seems to have fluctuated only from arid to semi-arid since the end of the Miocene with evidence of a humid period in the Kalahari in the late Pleistocene (Lancaster 1979, 1984). The northern clade was separated from the other three clades by the *Brachystegia* woodland and was not as influenced by the continual expansion and contraction of habitat caused by constant climatic changes.

A number of recent studies on large and small African mammals (Matthee and Robinson 1997; Arctander *et al.* 1999; Pitra *et al.* 2002; Alpers *et al.* 2004) have also described genetic divisions between southern Africa and eastern Africa showing similar patterns to that of the Helmeted Guineafowl.

The subspecific morphological differences separating these taxa appear to have occurred in the context of relatively shallow evolutionary separation. The possibility also exists that the regional morphological differences are largely ecophenotypic. Alternatively, the phenotypic geographic differentiation is indeed genetic but the nuclear genes underpinning this variation have evolved so rapidly over a narrow evolutionary timescale that the differences are not yet detectable in the mitochondrial genome (Ball *et al.* 1988). Another hypothesis is that geographical variation in local selection pressures act to maintain adaptive differences in migratory behaviour, morphological variation and, possibly other unknown traits in these birds. The alternative is that these phenotypic differences reflect plastic responses to environmental cues but this seems unlikely as other studies have shown heritable variation exists for other traits such as melanin-based plumage traits (Theron *et al.* 2001) and migratory behaviour (Berthold and Helbig 1992). There is also circumstantial evidence for geographically varying selection pressures on plumage variation in birds (Price 1998).

These findings are similar to other molecular studies of bird taxa, which have demonstrated that morphological and behavioural traits, such as plumage colouration, size and acoustic characters, do not always correlate with the evolutionary history of a group (e.g. song sparrow *Melospiza melodia* (Zink and Dittman 1993), bluethroat *Luscinia svecica* (Questiau *et al.* 1998), and common crossbill *Loxia curvirostra* (Questiau *et al.* 1999).

The genetic structuring apparent among the Helmeted Guineafowl populations showed some geographical consistency, but interestingly did not concur strongly with the previously recognised subspecies boundaries. It must be noted however, that the phylogenetic clades described in this study closely match groupings of operational taxonomic units (OTUs) in a cluster analysis of 704 *N. meleagris* specimens according to 22 morphological characters by Crowe (1978).

Even though there were no strong phylogenetic associations corroborating the subspecies designations, these phylogenetic reconstructions, which are based on mtDNA sequences alone, should be considered gene trees and not species trees until additional sequences from nuclear genes become available.

This study has highlighted the presence of a well-supported southern African group of Helmeted Guineafowl. The guineafowl of southern Africa can thus serve as an avian model for future phylogeographic studies attempting to explain patterns and processes that have shaped the biota of this region.

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Chapter 3

Phylogeography of the Helmeted Guineafowl in southern Africa

Phylogeography of the Helmeted Guineafowl in southern Africa

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Abstract

The differentiation of the mitochondrial DNA within and among populations of Helmeted Guineafowl (*Numida* meleagris) in southern Africa was assessed in this study. Analyses on the hypervariable control region included phylogenetic distance analysis, analysis of molecular variance (AMOVA) and nested clade analysis. There was evidence of genetic structuring between the populations inhabiting grassland biome and those occurring in the savanna and Karoo areas. Increased sampling throughout both areas should strengthen the support for this habitat split. There was no detection of any genetic differentiation between the two subspecies (*N. m. damarensis* and *N. m. coronata*) that were sampled in this study. The shallow divergences among the guineafowl in southern Africa suggest rapid or recent radiation. Low molecular variation could also be accounted for by the collapse of populations in the past due to disease, hunting, poisoning and the introduction of domesticated guineafowl.

Keywords: Helmeted Guineafowl, *Numida meleagris*, phylogeography, nested clade analysis, mtDNA, control region.

Introduction

The Helmeted Guineafowl (*Numida meleagris*) inhabit almost all open country terrain south of the Sahara (Crowe *et al.* 1986). They are especially found in areas of mixed savanna and cultivated land (Little 1997). All guineafowl species have highly terrestrial lifestyles, and fly only infrequently, normally either up onto their roosts at night, or in order to escape from predators, although in the latter case they often prefer to run off at great speed (Crowe *et al.* 1986). Despite their apparent reluctance to fly, guineafowl are highly mobile birds, and have been known to cover 30 to 40 km daily while foraging (Hastings Belshaw 1985). The legs and feet have three front toes and a single elevated hind one. The toes have strong claws and are well adapted to scratching for subterranean food. The wings are rather small and rounded and are designed for short bursts of rapid flight, enabling the birds to make a quick escape when danger threatens, but are quite unsuitable for any form of sustained flight. Their diurnal movements are dictated by a need to search for food and water (Crowe 2000b). The proximity to roosts, suitable cover and water holes, or other sources of drinking water, seems to be a major limiting factor in the distribution of guineafowl (Crowe *et al.* 1986; Crowe 2000b).

Although guineafowl are mobile, they lead very sedentary lives (Crowe 1978) and are rarely found further than 10km away from water, which would suggest that there would be a high level of genetic structuring among populations. Each major flock keeps to well defined territorial areas although there is some traffic of individual birds between flocks (Hastings Belshaw 1985). The recognition of two well-marked subspecies (*N. m. damarensis* and *N. m. coronata*) with close geographical distributions in Namibia and South Africa (Crowe 1978) lends strength to the view that there is a low level of gene flow between populations of guineafowl.

Modern studies of geographical variation emphasise molecular techniques, as investigators search for spatial patterns of genetic variation that can be interpreted in the context of evolutionary models and to prioritise areas for conservation (Avise 1994; Humphries *et al.* 1995; Moritz and Faith 1998; Smith *et al.* 2000). The populations of most, if not all, species show some levels of genetic structuring, which may be due to a variety of non-mutually exclusive processes. Environmental barriers, historical processes and life histories may all, to some extent, shape the genetic structure of populations (Tiedemann *et al.* 2000; Balloux and Lugon-Moulin 2002). The use of phylogenetic analyses of intraspecific variation in DNA was aptly termed "phylogeography" by Avise *et al.* (1987). Recent use of phylogeographic analyses of mtDNA sequences has made possible more detailed studies of historical biogeography (Avise 2000; Arbogast and Kenagy 2001). In particular one can examine phylogeographic patterns of mitochondrial DNA (mtDNA) variation, and evaluate the relative roles of gene flow, bottlenecks, and historical or ecological barriers in effecting spatial patterns (Zink 1996; Mock *et al.* 2002). For conservation planning to be successful, biologists

should try to preserve not only the pattern of biodiversity but also the evolutionary processes that generate and maintain it (Erwin 1991; Smith *et al.* 1993; Bulgin *et al.* 2003; Johnson *et al.* 2003).

The most commonly used molecular marker for phylogeographic studies has been animal mtDNA. Mitochondrial DNA has been used in many intraspecific phylogeography studies, because its high mutation rate allows researchers to distinguish recently diverged lineages. In particular, several authors (Randi *et al.* 2003; van den Bussche *et al.* 2003; Barrowclough *et al.* 2004; Eggert *et al.* 2004; Gay *et al.* 2004) have identified the control region as a useful tool to assess the extent of female gene flow between populations.

In this research, analyses of the 5' hypervariable domain of the mtDNA control region were used to assess the extent of mtDNA differentiation among populations of Helmeted Guineafowl, and subsequently to draw inferences on demographic processes and determine the degree of population genetic structuring.

Materials and Methods

Study area and sampling

The localities of all guineafowl sampled are presented in Table 3.1 along with the number of individuals sampled per population and the respective haplotypes determined. Geographical sampling sites are illustrated in Figure 3.1.

Due to the introduction of domesticated guineafowl of West African origin and subsequent introgression with naturally wild populations (Rossouw 1996; Walker 2000; Walker *et al.* in press) several birds sampled grouped into a putative domesticated/West African clade. Only the sequences of birds that grouped within the natural southern African clade were included in subsequent phylogeographic analyses. Some samples were excluded based on morphological evidence of hybridisation.

DNA extraction, PCR amplification and sequencing

DNA was extracted from all blood and tissue samples using a standard Proteinase K digestion followed by phenol/chloroform extraction (Sambrook *et al.* 1989). The digestion was performed in 500µl of extraction buffer (0.05M Tris-HCl, 0.001M EDTA·Na_2, 0.1M NaCl, 0.5%SDS) with 50µl Proteinase K (10µg/ml) (Roche Diagnostics). Samples were digested overnight at 55˚C followed by incubation for 1 hour at 37˚C with 60µl RNase A (1 mg/ml) (Roche Diagnostics). Thereafter samples were extracted twice with phenol and once with a chloroform:isoamyl alcohol (24:1) solution. Samples were then precipitated overnight at -20˚C in a solution containing 0.1 volumes 3M sodium acetate and 2 volumes of 96% Ethanol. The genomic DNA was finally pelleted in a desktop microcentrifuge at 14000rpm and resuspended in 50µl Sabax® water (Adcock Ingram). The 5' domain of the control region was amplified by polymerase chain reaction (PCR; Saiki *et al.* 1988) using the primers L16747 (Wenink *et al.* 1994) and H522 (Quinn and Wilson 1993).

Approximately 100ng of genomic DNA was used as template in a total PCR reaction of 50µl. In addition to the genomic DNA, the reaction mix which was ¼ strength contained: 2mM $MgCl_2$, 1 x reaction buffer, 0.2mM of each of the four nucleotides, 12.5 picamol of each primer and 1.5U of Super-therm® DNA polymerase (Southern Cross Biotechnology). A Geneamp® PCR System 9700 (Applied Biosystems) was used to cycle the reaction mix through the following conditions: denaturing at 94˚C for 2min; followed by 35 cycles of denaturing at 94˚C for 30 seconds, primer annealing at 52-56˚C for 30 seconds and elongation at 72˚C for 30 seconds; and finally an extended elongation period of 10 minutes at 72˚C. The PCR products were purified by precipitating with an ethanol and sodium acetate solution. Dye-terminator (Sanger *et al.* 1977) cycle sequencing was performed, using primers L16747 and H522 for the control region, with the BigDye DNA Ready Reaction sequencing kit (Applied Biosystems) in a Geneamp® PCR System

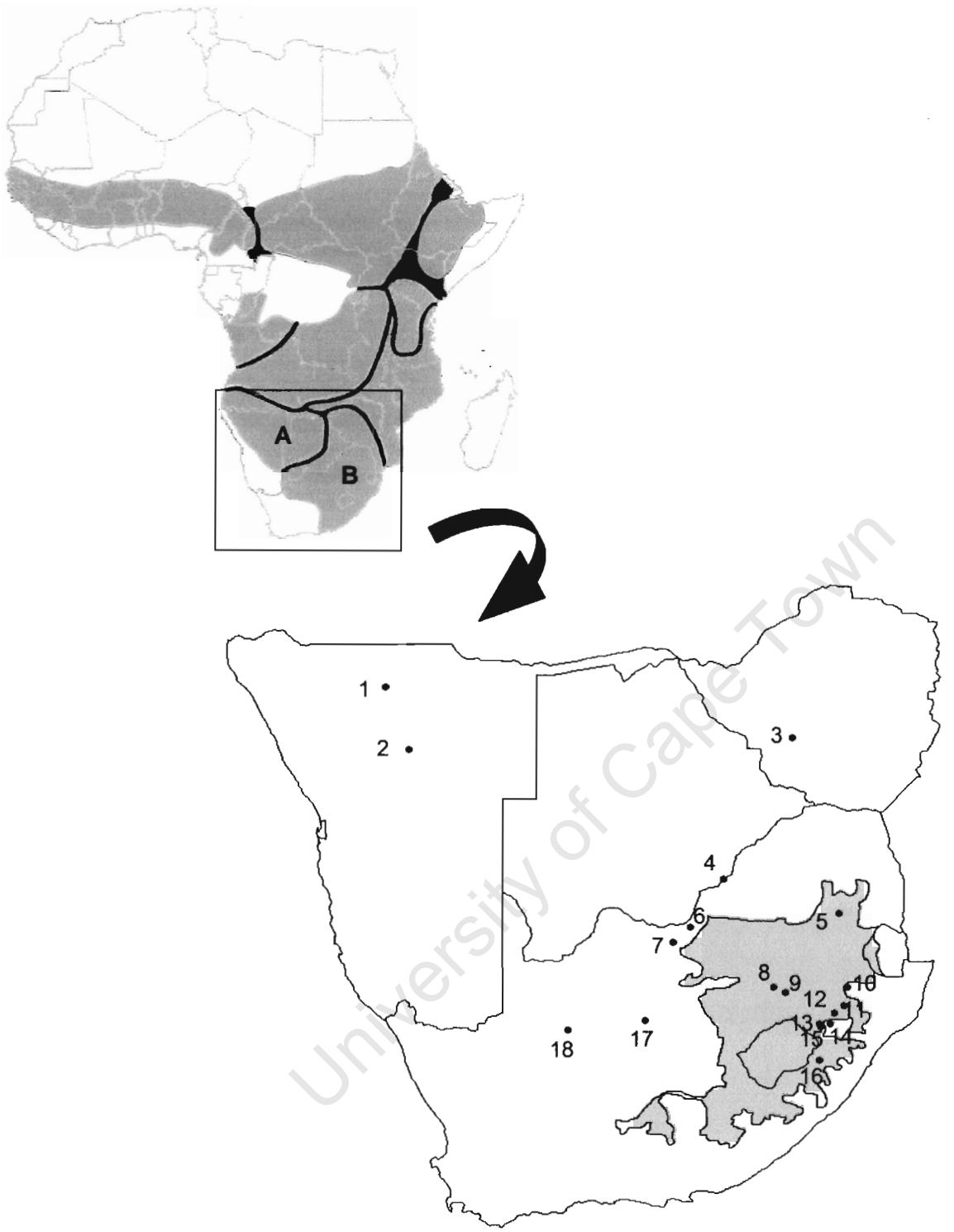

Figure 3.1. Collecting localities of *Numida meleagris* from southern Africa. The top left inset shows the distribution of *Numida meleagris* throughout Africa, **A**= *N. m. damarensis* **B**= *N. m. coronata.* The numbers correspond to the locality numbers in Table 3.1. 1-Etosha; 2-Waterberg; 3-Bulawayo; 4-Marico; 5-Dullstroom; 6-Mafikeng; 7-Setlagoli; 8-Petrus Steyn; 9-Reitz; 10-Utrecht; 11-Dundee; 12-Colenso; 13-Winterton; 14-Elandslaagte; 15-Spioenkop; 16-Underberg; 17-Rooipoort; 18-Groblershoop

- Extent of grassland distribution

9700 (Applied Biosystems). Thereafter, nucleotide sequences were determined through electrophoresis on an ABI 3100 automated sequencer (Applied Biosystems).

Sequence analysis

Heavy and light strand sequences for the control region were imported into Sequence Navigator version 1.0.1 (Applied Biosystems) where they were proofread for each sample. Consensus sequences of each sample were thereafter aligned using CLUSTAL X version 1.74 (Thompson *et al.* 1997). Phylogenetic relationships among the representative mtDNA haplotypes were estimated using the neighbour-joining method (Saitou and Nei 1987) carried out in PAUP* version 4.0b10 (Swofford 2000). Nodal support was assessed using 1000 bootstrap replications (Felsenstein 1985). The parameters for the neighbour-joining analysis were selected by hierarchical likelihood ratio testing using MODELTEST 3.06 (Posada and Crandall 1998), the AIC criteria was followed. Haplotype and nucleotide diversities were estimated using DnaSP version 3.51 (Rozas and Rozas 1999). Analysis of molecular variance (AMOVA; Excoffier *et al.* 1992), using 1000 permutation tests to estimate significance levels, and population differentiation via the estimation of pairwise F_{ST} values was examined using the program Arlequin 2.000 (Schneider *et al.* 2000).

The statistical significance of phylogeographic associations was tested using nested clade analysis. This was based on the phylogenetic relationships among haplotypes and the frequency of haplotypes in each population. A haplotype network with 95% probability based on parsimonious connections was constructed using the program TCS 1.13 (Clement *et al.* 2000) with the statistical approach developed by Templeton *et al.* (1992). The network was then converted into a nested clade design using standard nesting rules (Templeton *et al.* 1987; Templeton and Sing 1993). Nested contingency analysis and nested geographical distance analysis were implemented with GeoDis 2.0 (Posada *et al.* 2000) using 1000 permutations. The results of the nested geographical analysis were interpreted to determine if there was any relationship between genetic distance and geographic distance using Templeton's (2004) interpretive key.

Table 3.1. Geographic coordinates of all collecting localities of *Numida meleagris* in southern Africa analysed in the present study. The locality numbers correspond to those in Figure 3.1 and the haplotypes numbers correspond with those in Table 3.2.

	Locality	Country	Province	Geographic coordinates	No. of individuals	Haplotype no's.
1	Etosha	Namibia		18° 40' S 16° 30' E	1	23
2	Waterberg	Namibia		20° 32' S 17° 12' E	2	19, 24
3	Bulawayo	Zimbabwe		20° 10' S 28° 40' E	1	18
4	Marico	South Africa	Limpopo	24° 24' S 26° 38' E	3	5, 12, 26
5	Dullstroom	South Africa	Mpumalanga	25° 25' S 30° 06' E	2	2
6	Mafikeng	South Africa	North West	25° 50' S 25° 38' E	1	16
7	Setlagoli	South Africa	North West	26° 17' S 25° 07' E	2	4, 22
8	Petrus Steyn	South Africa	Free State	27° 38' S 28° 08' E	4	1, 2, 6
9	Reitz	South Africa	Free State	27° 48' S 28° 29' E	10	1, 2, 3, 27
10	Utrecht	South Africa	KwaZulu-Natal	27° 38' S 30° 20' E	2	13, 20
11	Dundee	South Africa	KwaZulu-Natal	28° 11' S 30° 15' E	6	7, 9, 11, 20
12	Colenso	South Africa	KwaZulu-Natal	28° 44' S 29° 50' E	1	9
13	Winterton	South Africa	KwaZulu-Natal	28° 49' S 29° 32' E	1	11
14	Elandslaagte	South Africa	KwaZulu-Natal	28° 24' S 29° 57' E	1	14
15	Spioenkop	South Africa	KwaZulu-Natal	28° 43' S 29° 31' E	1	10
16	Underberg	South Africa	KwaZulu-Natal	29° 48' S 29° 30' E	2	10, 11
17	Rooipoort	South Africa	Northern Cape	28° 38' S 24° 17' E	10	1, 7, 8, 15, 17, 21, 22, 25
18	Groblershoop	South Africa	Northern Cape	28° 54' S 21° 59' E	1	8

Results

Control region sequence analysis

Nuclear copies of mitochondrial genes (called numts) have been documented in a wide variety of organisms (Sorenson and Fleischer 1996; Zhang and Hewitt 1996; Quinn 1997; Sorenson and Quinn 1998) and there is a widespread concern over their effects on studies of molecular systematics and population biology. We are confident that all of the control region sequences obtained in this study are mitochondrial in origin. Almost all sample DNA was extracted from tissue and not blood, as blood is known to be prone to amplification of numts since it is poor in mtDNA (Quinn 1992; Arctander 1995; Sorenson and Fleischer 1996). Additionally we detected no evidence of multiple copies of the control region in any of our sequences suggesting that our primers had not amplified a mixture of mitochondrial and nuclear copies.

The sequence data comprised a 321 base pair fragment of the mitochondrial control region with 24 variable characters (Table 3.2) defining 27 haplotypes from the 51 individuals sampled. The localities of shared identical haplotypes are shown in Table 3.2. The mean nucleotide frequencies of the control region (A=30.2, C=27.2, G=13.5 and T=29.1) were similar to that of other avian 5' control regions sequenced (Baker and Marshall 1997) with a high A/T nucleotide content and a deficiency of G–nucleotides.

The best-fit model for the data, calculated by Modeltest (Posada and Crandall 1998), was the Tamura-Nei model of substitution (Tamura and Nei 1993) with a proportion of invariable sites equal to 0.8553. Pairwise estimates of the percentage sequence divergence Tamura-Nei + I and HKY85 (Hasegawa *et al.* 1985) are presented in Table 3.3. The sequence divergences among the 27 maternal haplotypes ranged from 0.32% to 5.41%.

The neighbour-joining tree (Figure 3.2) shows shallow geographical partitioning within the Helmeted Guineafowl. There is also a lack of correspondence with the current subspecific taxonomy (Crowe 1978) as haplotypes H19, H23 and H24 (Table 3.2) traditionally recognised as *N. m. damarensis* group together with samples of *N. m. coronata* (Figure 3.2). A shallow split of the 27 haplotypes into two groups is evident in Figure 3.2 although the support for this node is very weak (< 50%). Group A (Figure 3.2) consists of haplotypes H1 – H11 and group B (Figure 3.2) comprises haplotypes H12 – H27. The estimated sequence divergences among the 11 haplotypes of group A are small (0.32% - 2.10%; Tamura-Nei) with a mean divergence of 1.18% (SD 0.57%). Group B has more variable sequence divergences, ranging from 0.32% to 3.84% (Tamura-Nei), although generally they are also small (Mean 1.79%, SD 0.98%). Despite the lack of bootstrap support for the split of the two groups in the neighbour-joining tree (Figure 3.2), the sequence divergences between group A and group B are quite high (Mean 4.07%, SD 1.87%).

Table 3.2. Distribution of 27 observed mtDNA control region haplotypes from a sample of 51 Helmeted Guineafowl from 18 sampling localities. The vertical numbers indicate the positions of variable nucleotides within the 321 bp sequence. Dots indicate the same nucleotide is present as in haplotype 1. The number of individuals for each haplotype from a population are given, with the numbers in parentheses () following a population name corresponding to the locality numbers in Figure 3.1. Haplotypes in bold are *N. m. damarensis*, all other haplotypes are *N. m. coronata.*

Haplotype No.	Variable Positions 111111111111111112222 223711222234445556990001 297009247871572798231582	No. of individuals	Populations (Locality No.)
H1	CTGCCATTTCTTCCTATTTCCCTT	3	Reitz (9)
		2	Petrus Steyn (8)
		1	Rooipoort (17)
H2	.....C....	5	Reitz (9)
		2	Dullstroom (5)
		1	Petrus Steyn (8)
H3	.....T.....	1	Reitz (9)
H4	.....C.....	1	Setlagoli (7)
H5	.....T.....C.....	1	Marico (4)
H6	....T.....CT....	1	Petrus Steyn (8)
H7	.....C.....	1	Rooipoort (17)
		1	Dundee (11)
H8	.....G.....CG.....	1	Rooipoort (17)
		1	Groblershoop (18)
H9	.....C.....T...	1	Colenso (12)
		1	Dundee (11)
H10	.....C....T...C	1	Spioenkop (15)
		1	Underberg (16)
H11	.....C.....C....T...C	1	Underberg (16)
		1	Dundee (11)
		1	Winterton (13)
H12	.....C.C..TCG....T...	1	Marico (4)
H13	.....C.C.C...C....TT.C.	1	Utrecht (10)
H14	.C....C.C.C...C..C....C.	1	Elandslaagte (14)
H15	.....C.C..TCG....T.C.	2	Rooipoort (17)
H16	.....C.C..TCG?...T.C.	1	Mafikeng (6)
H17	.....C.C..TCG....TTC.	2	Rooipoort (17)
H18	.....C.C..TCGC...TTCC	1	Bulawayo (3)
H19	**.....C.C..TC.....T.C.**	**1**	**Waterberg (2)**
H20	T.A.....C.C..TC.....T.C.	3	Dundee (11)
		1	Utrecht (10)
H21	..A.T...C.C..TC.....T.C.	1	Rooipoort (17)
H22	..A.T...C.C.TTC.....T.C.	1	Rooipoort (17)
		1	Setlagoli (7)
H23	**.....C.C..TC..C..TTC.**	**1**	**Etosha (1)**
H24	**.....G..CTC..TC.....C.**	**1**	**Waterberg (2)**
H25	.....CTCC.TC.C...T.C.	1	Rooipoort (17)
H26	.....CTCC.TC.C...TTC.	1	Marico (4)
H27	.....CTC..TC.C.CTT...	1	Reitz (9)

Table 3.3. Pairwise estimates of percentage Tamura-Nei + I (0.6839) sequence divergence among 27 haplotypes (below the diagonal) and percentage HKY85 sequence divergence (above the diagonal). Haplotype numbers correspond to Table 3.2 and those in Fig. 3.2. Within-group divergences are highlighted. The light grey block indicates group A and the dark grey block indicates group B in Figure 3.2.

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20	H21	H22	H23	H24	H25	H26	H27
H1		0.31	0.31	0.31	0.63	0.94	0.31	0.94	0.63	0.94	1.26	1.91	2.23	2.23	2.23	2.24	2.56	3.23	1.91	2.56	2.56	2.89	2.56	2.23	2.89	3.23	2.89
H2	0.32		0.63	0.63	0.94	0.63	0.63	1.26	0.94	1.26	1.58	2.23	2.56	2.56	2.56	2.57	2.89	3.56	2.23	2.89	2.89	3.23	2.89	2.56	3.23	3.56	2.56
H3	0.32	0.65		0.63	0.94	1.26	0.63	1.26	0.94	1.26	1.58	1.58	2.56	2.56	1.91	1.91	2.23	2.89	1.58	2.23	2.23	2.56	2.23	1.91	2.56	2.89	2.56
H4	0.32	0.65	0.65		0.31	1.26	0.63	1.26	0.94	1.26	1.58	2.23	2.56	1.91	2.56	2.57	2.89	3.56	2.23	2.89	2.89	3.23	2.23	2.56	3.23	3.56	3.23
H5	0.65	0.99	0.99	0.32		1.58	0.94	1.58	1.26	1.58	1.91	2.56	2.89	2.23	2.89	2.90	3.23	3.90	2.56	3.23	2.56	2.89	2.56	2.89	3.56	3.90	3.56
H6	0.99	0.65	1.34	1.34	1.71		1.26	1.90	1.58	1.26	1.58	2.89	2.56	3.23	3.23	3.24	3.56	4.24	2.89	3.56	3.56	3.90	3.56	3.23	3.90	4.24	2.56
H7	0.32	0.65	0.65	0.65	0.99	1.34		0.63	0.31	0.63	0.94	1.58	1.91	1.91	1.91	1.92	2.23	2.89	1.58	2.23	2.23	2.56	2.23	1.91	2.56	2.89	2.56
H8	0.96	1.29	1.29	1.29	1.64	1.99	0.64		0.94	1.26	1.58	1.58	2.55	2.55	1.90	1.91	2.23	2.88	2.23	2.88	2.88	3.21	2.88	2.55	3.21	3.55	3.21
H9	0.65	0.99	0.99	0.99	1.34	1.71	0.32	0.96		0.94	1.26	1.26	1.58	2.23	1.58	1.59	1.91	2.56	1.26	1.91	1.91	2.23	1.91	2.23	2.23	2.56	2.23
H10	0.99	1.34	1.34	1.34	1.71	1.34	0.65	1.29	0.99		0.31	2.23	1.91	2.56	2.56	2.57	2.89	2.89	2.23	2.89	2.89	3.23	2.89	2.56	3.23	3.56	2.56
H11	1.34	1.71	1.71	1.71	2.10	1.71	0.99	1.63	1.34	0.32		1.91	1.58	2.23	2.23	2.24	2.56	2.56	1.91	2.56	2.56	2.89	2.56	2.23	2.89	3.23	2.23
H12	2.03	2.42	1.66	2.42	2.82	3.25	1.66	1.67	1.31	2.42	2.03		1.58	2.23	0.31	0.31	0.63	1.26	0.63	1.26	1.26	1.58	1.26	1.58	1.58	1.91	1.58
H13	2.50	2.92	2.92	2.92	3.37	2.92	2.10	2.76	1.71	2.10	1.71	1.66		1.26	1.26	1.27	1.58	2.23	0.94	1.58	1.58	1.91	1.58	1.91	1.91	2.23	1.91
H14	2.50	2.93	2.92	2.10	2.50	3.83	2.10	2.76	2.50	2.93	2.50	2.42	1.34		1.91	1.93	2.23	2.89	1.58	2.23	2.23	2.56	1.58	1.91	2.56	2.89	3.23
H15	2.42	2.82	2.03	2.82	3.24	3.69	2.03	2.04	1.66	2.82	2.42	0.32	1.31	2.03		0.00	0.31	0.94	0.31	0.94	0.94	1.26	0.94	1.26	1.26	1.58	1.91
H16	2.43	2.84	2.03	2.84	3.26	3.70	2.04	2.05	1.67	2.84	2.43	0.32	1.32	2.05	0.00		0.31	0.95	0.31	0.94	0.94	1.26	0.95	1.27	1.27	1.59	1.92
H17	2.82	3.24	2.42	3.24	3.69	4.16	2.42	2.44	2.03	3.24	2.82	0.65	1.66	2.42	0.32	0.32		0.63	0.63	1.26	1.26	1.58	0.63	1.58	1.58	1.26	2.23
H18	3.69	4.15	3.24	4.15	4.65	5.17	3.24	3.27	2.82	3.24	2.82	1.34	2.42	3.25	0.99	1.00	0.65		1.26	1.91	1.91	2.23	1.26	2.23	1.58	1.26	2.23
H19	2.10	2.50	1.71	2.50	2.92	3.37	1.71	2.37	1.34	2.50	2.10	0.64	0.99	1.71	0.32	0.32	0.64	1.31		0.63	0.63	0.94	0.63	0.94	0.94	1.26	1.58
H20	2.82	3.24	2.42	3.24	3.69	4.16	2.42	3.10	2.03	3.24	2.82	1.31	1.66	2.42	0.98	0.98	1.31	2.00	0.64		0.63	0.94	1.26	1.58	1.58	1.91	2.23
H21	2.82	3.24	2.42	3.24	2.82	4.16	2.42	3.10	2.03	3.24	2.82	1.31	1.66	2.42	0.98	0.98	1.31	2.00	0.64	0.65		0.31	1.26	1.58	1.58	1.91	2.23
H22	3.25	3.69	2.82	3.69	3.25	4.66	2.82	3.51	2.42	3.69	3.24	1.65	2.03	2.82	1.31	1.31	1.65	2.37	0.97	0.99	0.32		1.58	1.91	1.91	2.23	2.56
H23	2.92	3.37	2.50	2.50	2.92	4.33	2.50	3.17	2.10	3.37	2.92	1.31	1.71	1.71	0.97	0.97	0.64	1.31	0.65	1.31	1.31	1.66		1.58	1.58	1.26	2.23
H24	2.42	2.82	2.03	2.82	3.24	3.69	2.03	2.71	2.42	2.82	2.42	1.65	2.03	2.03	1.30	1.31	1.65	2.37	0.97	1.65	1.65	2.00	1.66		1.26	1.58	1.91
H25	3.37	3.83	2.92	3.83	4.33	4.85	2.92	3.60	2.50	3.83	3.27	1.66	2.10	2.93	1.31	1.32	1.66	1.66	0.99	1.66	1.66	2.03	1.71	1.31		0.31	1.26
H26	3.83	4.33	3.37	4.33	4.85	5.41	3.37	4.05	2.92	4.33	3.83	2.03	2.50	3.37	1.66	1.67	1.31	1.31	1.34	2.03	2.03	2.42	1.34	1.66	0.32		1.58
H27	3.37	2.92	2.93	3.83	4.33	2.93	2.92	3.60	2.50	2.92	2.50	1.66	2.10	3.84	2.03	2.04	2.42	2.42	1.71	2.42	2.42	2.82	2.50	2.03	1.34	1.71	

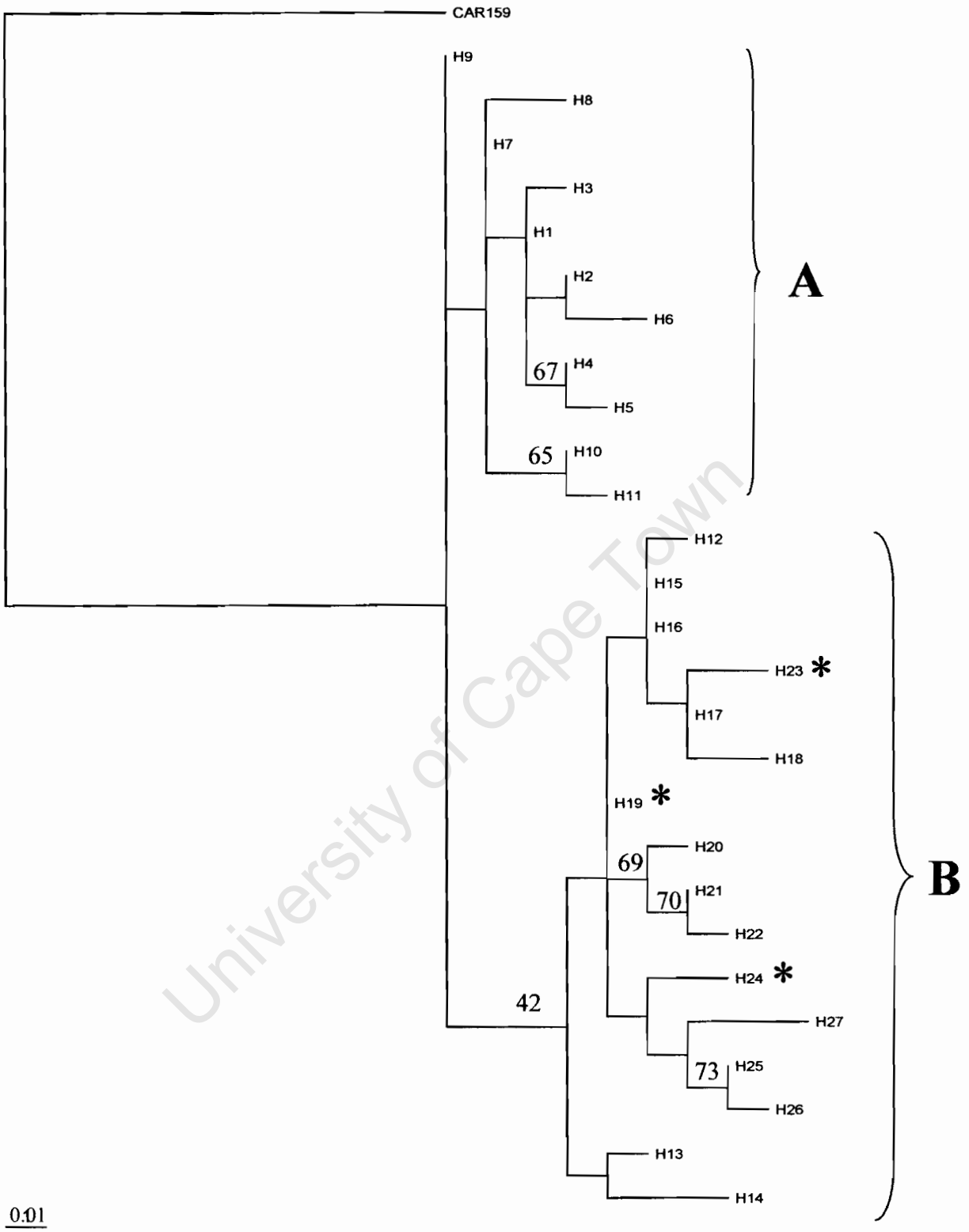

Figure 3.2. Neighbour-joining phylogram based on sequence divergence among mitochondrial DNA control-region sequences within *Numida meleagris* from southern Africa. The two groupings 'A' and 'B' correspond to those in Table 3.3. Bootstrap confidence levels are given at nodes where it is >50% (1000 reps). An individual *N. m. meleagris* from the Central African Republic was used as an outgroup. Haplotypes indicated by the symbol * represent the subspecies *N. m. damarensis*.

Analysis of molecular variance (AMOVA)

Molecular variation between the two morphological subspecies, *N. m.* coronata and *N. m.* damarensis, was not significantly different from that expected from random data (V_a = 0.006, P = 0.309, Φ_{ct} = 0.012) with the greatest proportion of variation found within populations (85.5%, V_c = 0.417, P = 0.001, Φ_{st} = 0.012). The variation amongst populations within the subspecies accounted for 13.29% of the variance (V_b = 0.065, P = 0.001, Φ_{sc} = 0.135). A geographical split seemed likely from the tree topology in Figure 3.2. Group B contained haplotypes mostly exhibited in localities 1, 2, 3, 4, 6, 7, 17 and 18 with group A comprising localities 5, 8, 9, 10, 11, 12, 13, 14, 15 and 16 (Figure 3.1). The AMOVA based on these geographical groups delineated in Figure 3.2 showed that the variance between the two groups, like the variance between the two subspecies, was not significantly different from random and only accounted for 4.08% of the variation observed (V_a = 0.02, P = 0.075, Φ_{ct} = 0.041). Variance within sampling localities was responsible for 84.81% of the variation (V_c = 0.41, P = 0.001, Φ_{st} = 0.152), whilst the remaining 11.11% was due to variance among sampling localities within the two groups (V_b = 0.05, P = 0.001, Φ_{sc} = 0.116). The nucleotide diversity for group A was 0.78% and for group B 1.32% with a total diversity for all samples of 1.76%. The total haplotype diversity was 0.95 with group A having a haplotype diversity of 0.88 and group B one of 0.95 indicating that both groups have a high incidence of locality-specific haplotypes. The small sample size may account for this high haplotype diversity value and we would expect this to reduce with greater sampling.

On closer inspection it appeared that this divide between the two groups of guineafowl might not be due to some physical boundary but could possibly be due to habitat restrictions. The guineafowl with a northerly distribution inhabit Nama Karoo and Savanna vegetation types while the guineafowl with a more central and eastern distribution occupy Grassland vegetation. However, some of the localities with an eastern distribution occupy savanna and were thus grouped together with grassland localities in the first AMOVA test (for example sampling localities 10 and 14, see Figure 1 for distribution of Grassland). Therefore another AMOVA was performed but this time all localities believed to occur within the grassland biome were grouped together and all those localities thought to occur in Karoo and savanna were grouped together.

The amount of variance provided by these newly defined habitat based groupings was still very small (4.45%). Contrary to the initial groupings, these groups were significantly different from that expected from random sampling (V_a = 0.022, P = 0.041, Φ_{ct} = 0.044) with variance within sampling localities still creating the majority of variation at 84.73% (V_c = 0.42, P = 0.001, Φ_{st} = 0.153). However, as there are three samples from two localities representing a second subspecies (*N. m. damarensis*) morphologically distinct from the subspecies that occurs in South Africa (*N. m. coronata*) and the fact that there is a large geographical distance separating the sampling sites in

this study (Figure 3.1), it is possible that the significance of the differences between groups is a reflection of sampling bias. In order to test this, a separate AMOVA was performed excluding the samples of *N. m.* damarensis obtained from Namibia. The results showed that although the variance between the habitat groups in South Africa was similar to that of southern Africa, 4.32%, the groupings were no longer significantly different from random expectations (V_a = 0.022, P = 0.103, Φ_{ct} = 0.043). Hence, the samples from Namibia accounted for the significant variance values detected between habitat types within southern Africa. Greater sampling of *N. m. coronata* in the savanna habitat might even out the sampling bias and allow testing of the significance of the genetic differentiation between guineafowl populations from the two vegetation types.

Nested clade analysis

A haplotype network was constructed by the program TCS (Clement *et al.* 2000). Connections of a maximum of seven steps fell within the 95% confidence interval using parsimony (Figure 3.3a). Figure 3 shows the nested clade design of this haplotype network according to Templeton (2004). Twelve clades contained significant D_n, D_c or I-T distances, which resulted in a rejection of the null hypothesis that the haplotypes are distributed at random with respect to geography: three of the one step clades (1-1, 1-2 and 1-8), six of the two step clades (2-2, 2-3, 2-6, 2-7, 2-8 and 2-9), two of the three step clades (3-1 and 3-2) and the total cladogram (Figure 3.4). The chains of inference from these results are listed in Table 3.4. Restricted gene flow with isolation-by-distance was inferred from haplotypes in clade 1-1 (geographically from localities 4 and 7; population numbers correspond with those in Figure 1), clade 1-3 (localities 7 and 17), and clade 1-8 (localities 6 and 17). Contiguous range expansion was inferred from the haplotypes nested in clade 2-2 (localities 7, 10, 11 and 17). The one step clades nested within 2-6, led to an inference of restricted gene flow with isolation-by-distance (localities 10 and 14). It was inferred that there was contiguous range expansion for both clade 3-1 (localities 1, 2, 4, 7, 9, 10, 11 and 17) and clade 3-2 (localities 3, 4, 6 and 17). Restricted gene flow/dispersal but with some long-distance dispersal was inferred from the total cladogram over the entire geographical sampling area. Inconclusive outcomes were realised for clades 2-3, 2-7 and 2-8, while clade 2-9 revealed inadequate geographical sampling.

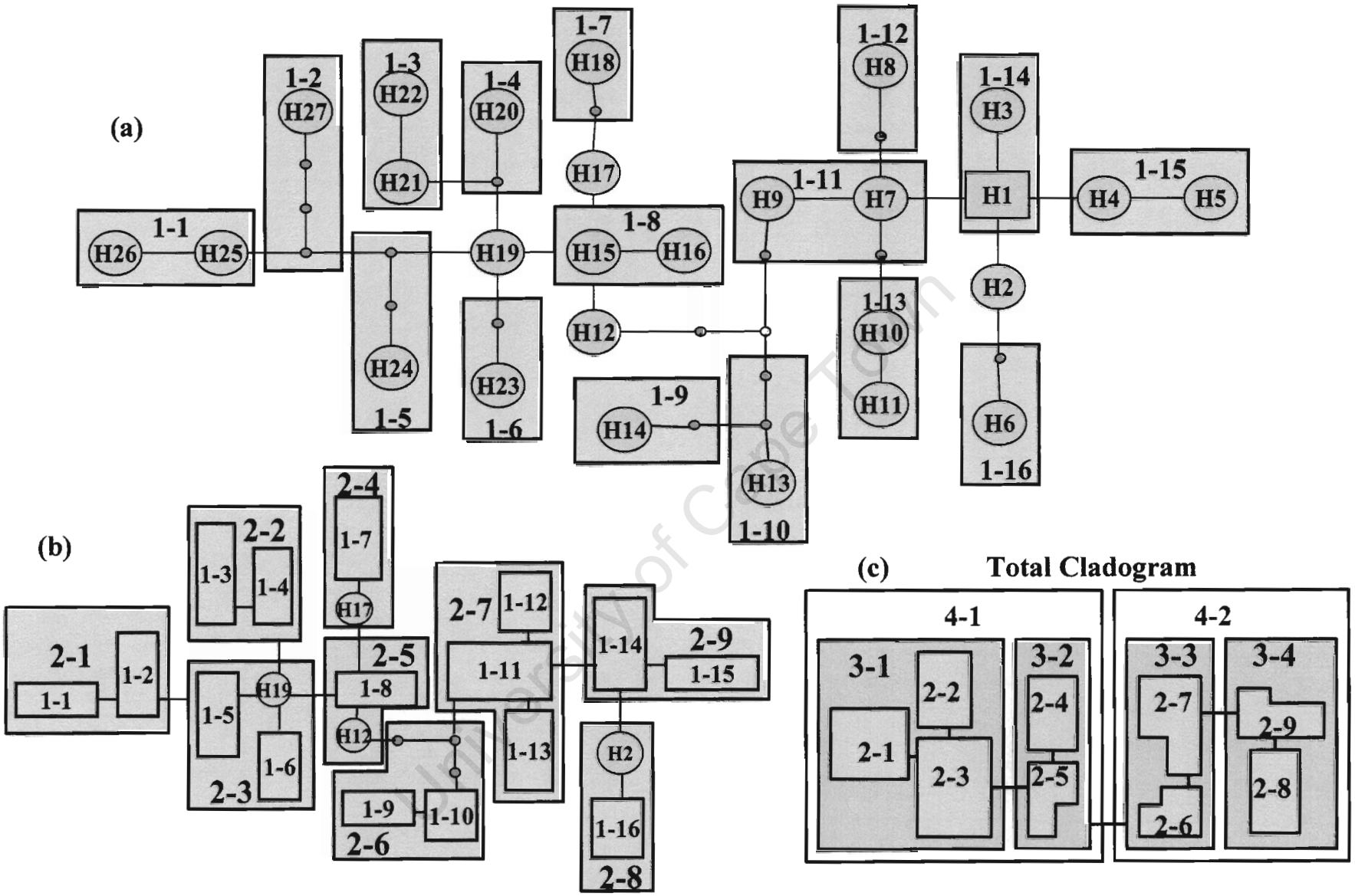

Figure 3.3 The estimated cladograms at the 95% confidence level and associated nested design for the mtDNA haplotypes found in *N. meleagris* from southern Africa. Haplotype states are designated with an **H** number and correspond to those presented in Table 3.2. Necessary intermediate haplotype statesthat were not present in the samples are indicated by a "dot". Each solid line represents a single mutational change that interconnects twohaplotype states that has a probability greater than 95%. One-step clades are boxed and labeled "1-x" where x is a number assigned to identify the clade. Two-step clades are labeled "2-x", three-step clades "3-x" and the Total Cladogram consists of the two four-step clades, 4-1 and 4-2.

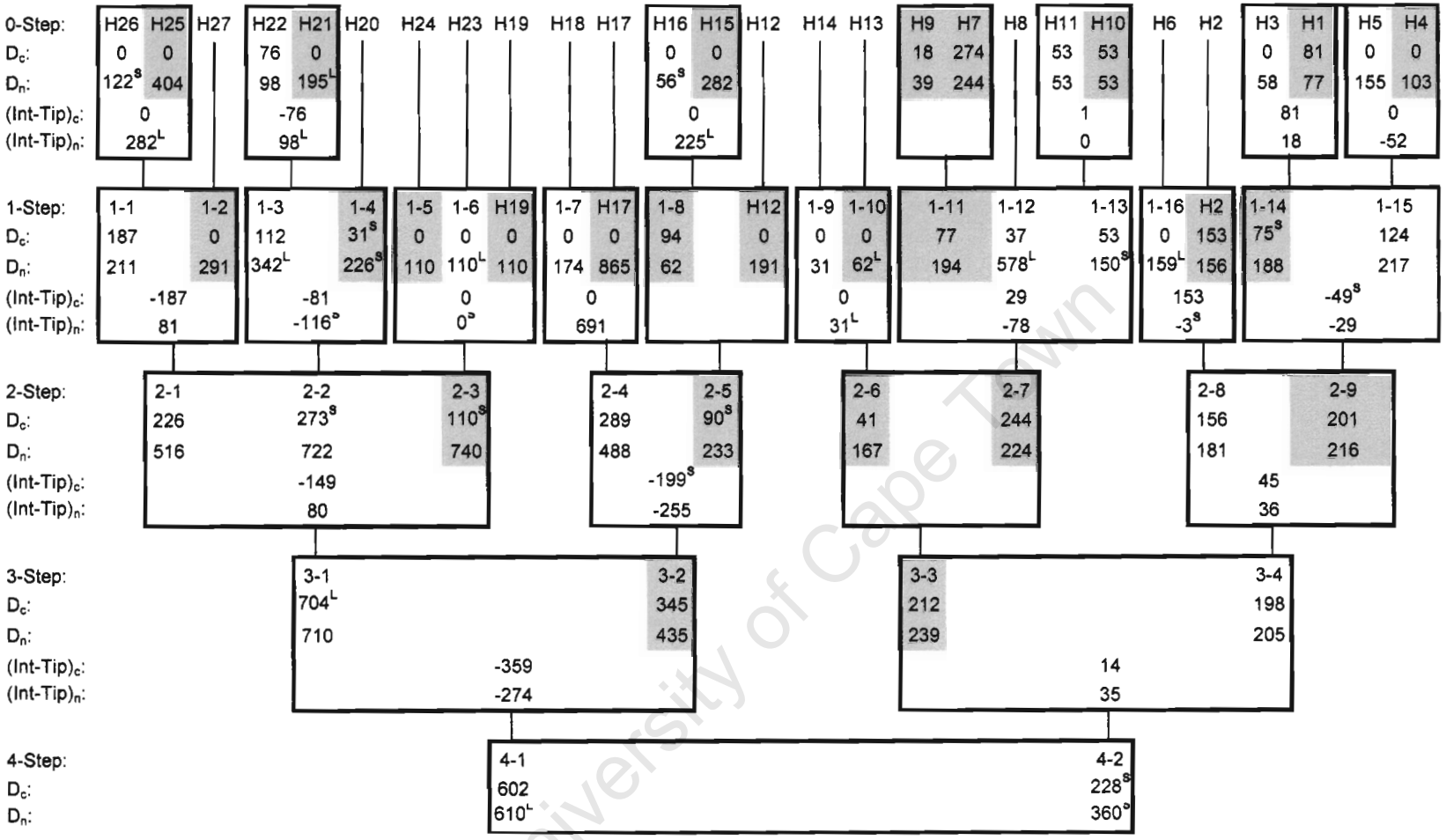

Figure 3.4. Results of the nested cladistic analysis of geographical distance for the mtDNA haplotypes of *N. meleagris*. The haplotype designations are given at the top and are boxed together to reflect the one-step nested design given in Figure 3.3. Higher level clade designations are given as one moves down the figure, with boxed groupings indicating the nesting structure. Immediately below each clade designation are the clade and nested clade distances respectively. An "S" superscript indicates the distance is significantly small at the 5% level, and an "L" indicates that it is significantly large. For nested clades in which the tip/interior status is known and for which both tips and interiors exist within the same nesting group, the clade name and distances are shaded for interior clades and are left unshaded for tip clades. At the bottom of the boxes that indicate the nested groups containing both tip and interior clades, the lines indicated by the symbols "(Int-Tip)c" and "(Int-Tip)n" give the average difference in distances between interior clades and tip clades within the nested group for clade distances and nested clade distances respectively.

Table 3.4. Inference chain on the results given in Figure 3.4. Only those clades that resulted in a rejection of the null hypothesis are included in this table.

Clade	Chain of Inference	Inference
Haplotypes nested in 1-1	1-19-20-2-11-17-4 NO	Restricted gene flow with isolation by distance
Haplotypes nested in 1-3	1-2-11-17-4 NO	Restricted gene flow with isolation by distance
Haplotypes nested in 1-8	1-19-2-11-17-4 NO	Restricted gene flow with isolation by distance
One-step clades nested in 2-2	1-19-2-11-12 NO	Contiguous range expansion
One-step clades nested in 2-3	1-2-11-17 NO	Inconclusive outcome
One-step clades nested in 2-6	1-19-20-2-11-17-4 NO	Restricted gene flow with isolation by distance
One-step clades nested in 2-7	1-2-11-17 NO	Inconclusive outcome
One-step clades nested in 2-8	1-2-11-17 NO	Inconclusive outcome
One-step clades nested in 2-9	1-19-20 NO	Inadequate geographical sampling
Two-step clades nested in 3-1	1-2-11-12 NO	Contiguous range expansion
Two-step clades nested in 3-2	1-2-11-12 NO	Contiguous range expansion
Four-step clades nested in Entire cladogram	1-2-3-5-6-7 YES	Restricted gene flow/dispersal but with some long-distance dispersal

Discussion

The analyses indicated a split of the Helmeted Guineafowl in southern Africa into two groups. These groups were believed to correspond with geographical locality, with guineafowl occurring in the northern and western parts of the distribution forming one group (group B Figure 3.2) and the guineafowl inhabiting the eastern parts of South Africa forming another group (group A Figure 3.2). Phylogeographical structure was detected among the sampling localities of Helmeted Guineafowl in southern Africa. Almost all of the structure was found to occur within group B (Figure 3.2), also corresponding with the savanna and Karoo dwelling guineafowl. The inferences determined for most of the significantly structured clades (Table 3.3) were of restricted gene flow with isolation by distance. We might expect to find this pattern in a sedentary animal whose dispersal patterns are limited in the search for food and water. Contiguous range expansion is also inferred for a number of clades (Table 3.3). Some of this inferred range expansion (i.e. clade 2-2) is occurring between localities that are geographically very distant from one another. The sampling localities in the middle of this expansion did not support the inference of contiguous range expansion in this region. The inferred range expansion in clades 3-1 and 3-2 seems more probable as the included sampling localities occur in a similar habitat and with a northerly distribution, but any conclusions drawn from this should be done so with caution because of large expanses of unsampled territory between the sample localities in this study. When the sampling is sparse in an area, it becomes impossible to discriminate between short and long-distance movements. Hence, strong inferences about the forces that explain the geographical distribution of genetic variation require adequate sampling (Templeton *et al.* 1995).

The shallow divergences in the tree (Figure 3.2) suggest that the Helmeted Guineafowl in southern Africa had a rapid radiation or have only recently radiated. This could also be explained by the dramatic expansion, both in range and population size, over the last century due to translocations by humans and the ability of guineafowl to exploit areas where low-level agriculture activities are conducted in a mosaic of natural vegetation types as well as urban landscapes (Little 1997). In particular, the guineafowl in group A (Figure 3.2) have relatively low variation within localities with 29 individuals having 11 haplotypes. The majority of samples in this group occur in the grassland biome which is what we might expect as history would suggest that this group would be more likely to have less variation. This is because since the 1980s, guineafowl populations have collapsed, in some cases to local extinction (Pero and Crowe 1996), in the KwaZulu-Natal province (South Africa). A number of factors have been attributed to having caused this, including habitat fragmentation, disease, illegal hunting and poisoning with pesticides (Pero and Crowe 1996; Crowe 2000a). Another potential threat to populations of guineafowl is the introduction of domesticated guineafowl into wild populations as the interbreeding between domestic and wild

birds might undermine the ability of their offspring to survive in the wild (Hastings Belshaw 1985; Wolff and Milstein 1987; Crowe 2000).

Sampling cannot be ruled out as having a major influence on the results, as the localities in the savanna-karoo are distant from one another and the sample size from each locality was very small. Although the sampling is not adequate enough to detect the effects of possible habitat fragmentation, we should not rule out the possibility that it may have had an impact on the guineafowl populations. Empirical studies have shown that habitat fragmentation can reduce genetic variability by decreasing local effective population size and by restricting gene flow (e.g. (Harrison and Hastings 1996; Caizergues *et al.* 2003). Reduced genetic variability resulting from habitat fragmentation has been reported in other avian species, including the blue grouse *Dendragapus obscurus* (Barrowclough *et al.* 2004), the sage grouse *Centrocercus urophasianus* (Oyler-McCance *et al.* 1999) and the greater prairie chicken *Tympanuchus cupido* (Bouzat and Johnson 2004). The latter two studies did, however, deal with extreme situations in which the genetically impoverished populations were isolated and had suffered extreme reduction in size.

There is evidence from this study of reasonable gene flow among populations as a result of female dispersal even though they are reported to be more sedentary than males (Crowe pers. comm.). The use of nuclear rather than mitochondrial markers will give a better representation of the gene flow caused by male biased dispersal and might show less population differentiation as some other studies have found (Kim *et al.* 1998; Wilmer *et al.* 1999; Piertney *et al.* 2000; Wirth and Bernatchez 2001).

The genetic structuring apparent among the Helmeted Guineafowl localities showed some geographical consistency, but interestingly did not concur strongly with the previously recognised subspecies boundaries. Little support was found for a population structure based on the recognised subspecies in AMOVA using mtDNA, more variance was found within subspecies groups than between them. This might be an artefact of inadequate sampling of the subspecies *N. m. damarensis*, only represented in this study by three samples but could reflect the lack of genetic differentiation between the two subspecies.

There is evidence of historical gene flow between the subspecies but not enough time has elapsed for lineage sorting between these geographically close subspecies. As limited gene flow between populations would be sufficient to prevent spatial heterogeneity in gene frequencies, the high level of private alleles suggests that the life-history characteristics of the species may lead to the detection of geographical structure at a much finer level of resolution than provided by the sampling and marker used in the present study. The apparent lack of differentiation may not be as a result of contemporary gene flow but rather due to the recent divergence of populations and

hence substantial sharing of ancestral variation. Genetic similarity between recently diverged taxa can be due to retained ancestral polymorphism and result in misleadingly high estimates of gene flow and a lack of phylogenetic distinctiveness (Bulgin *et al.* 2003). Specifically it can take many generations for completely isolated populations with large effective sizes to reach genetic equilibrium that will result in measures of differentiation reflecting the true level of genetic isolation experienced by two populations (Neigel and Avise 1986; Whitlock and McCauley 1999). This phenomenon may explain the high level of genetic similarity between the subspecies of the guineafowl.

Future work should focus on sampling throughout the entire distribution of both *N. m. damarensis* and *N. m. coronata*, paying particular attention to the suggested hybrid zone between the two subspecies (Crowe and Snow 1978) in order to determine the extent of gene flow between the two taxa and structuring among populations.

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Conclusions

The molecular variation among the present dataset of six subspecies is incongruent with the morphological variation currently used to delineate subspecies of *Numida meleagris*. From the present sampling of six subspecies, there is support for only four distinct clades at the molecular level. These clades appear to be geographically determined, occurring in northern, eastern, West and southern Africa. The reason for this distribution seems to have been the cyclical expansion and contraction of the *Brachystegia* woodland during the Pleistocene caused by constant fluctuations in climate. The southern African clade is the most well supported and clearly defined of the four clades, apparently escaping much of the habitat instability that affected Helmeted Guineafowl in other areas of Africa. In order that we might understand the processes that have caused these observed patterns, thorough sampling of all subspecies, throughout their distribution ranges needs to be undertaken. Of particular importance is the sampling of *N. m. marungensis*, which is geographically central to all of the subspecies and shares zones of intergradation with five other subspecies.

It does appear that there is some link between genetic and geographical structuring of the Helmeted Guineafowl in southern Africa. This structuring seems to be caused by the habitat boundary between grassland and savanna-karoo. There is particularly low molecular variation among individuals inhabiting the grassland biome, which might be as a result of previous population collapses in this area. Increased sampling of guineafowl around the habitat boundary might prove invaluable in clarifying whether the habitat variation is indeed responsible for the population structuring. The sampling of the two, well-marked, morphologically distinguished subspecies represented in the phylogeographic study did not show any significant genetic difference between the two. This is most likely due to the fact that there hasn't been complete lineage sorting between the two subspecies. Increased sampling, as well sampling along the suggested intergradation zone, might show some early molecular signs of differentiation.

Molecular techniques have shown that the Helmeted Guineafowl is a good avian model for studies at the intraspecific level, particularly for inferring processes that occurred in Africa during the Pleistocene period. There is also much scope for further studies into the structuring among populations within South Africa as well as the genetics of the intergradation zone that separates these morphologically distinct subspecies from one another.

Appendix 2.1

Sequence comparisons of 322 base pairs of the 5' end of the mitochondrial DNA control region for 18 individuals of *Numida meleagris*. Dots (.) indicate identity with the Uganda1 sequence and question marks (?) indicate missing nucleotide data. Crested = *Guttera pucherani* and Vulturine = *Acryllium vulturinum*.

```
[                  10         20         30         40         50         60     ]
[                   *          *          *          *          *          *     ]
Uganda1       TTATGGTACCGGTACTATATACTACATACATACTAAGCCCATATATATGTAAACGGACATAAATA
Uganda2       ............................................................
Malawi1       ........................T...TG.................................C.
Kenya1        ........................T...TG.............................G..C.
Angola        ......................T......TG......A.....................
Reitz         ........................T...TG.................................C.
Groblershoop  ........................T...TG.................................C.
Zimbabwe      ........................T...TG.................................C.
Namibia1      ........................T...TG.................................C.
Dundee        ......................T..T...TG......A.....................C.
Namibia2      ........................T...TG.................................C.
Harrods       ......................T..T...TG......A.....................
Malawi2       ......................T......TG......A.....................
Underberg     ........................T...TG.................................C.
Zambia        ........................T...TG.................................C.
Kenya2        ..............................................A.....................C.
CAR1          ..........A.............T...?.....A.....................
CAR2          ..........A.............T..........A.....................
CRESTED1      C.......................T....G......A.........C.................C.
CRESTED2      ......................T.GT....G......A.........C.................C.
VULTURINE     ......................T.G..C..G......A............C..............
```

```
[               70         80         90        100        110        120        130]
[                *          *          *          *          *          *          *]
Uganda1       CCTCCACCCCATTCCTCCCAAATGTACTAAAACATGTAATGCTTCCAGACATAAACTATAGTCCA
Uganda2       ............................................................
Malawi1       ..............................G...........T.........T....ACT..
Kenya1        ..............................G.....................T.....CT..
Angola        ............................G...G.........C..G........T....A....
Reitz         .....T........................G.........C.T..............A..T.
Groblershoop  .....T........................G.........C.T............G.A..T.
Zimbabwe      .....T........................G.........C.T..............AC.T.
Namibia1      .....T........................G.........C.T..............AC.T.
Dundee        .....T........................G.........C.T..............AC.T.
Namibia2      .....T........................G.........C.T..............AC.T.
Harrods       ............................G...G............G........T....AC...
Malawi2       ............................G...G.........C..G........T....A....
Underberg     ..............................G...........T.........T....ACT..
Zambia        ..............................G...........T.........T....ACT..
Kenya2        ...T..........................................................AC...
CAR1          ..............................................................C.......
CAR2          ..............................................................C.......
CRESTED1      .....T......................G..T..........C.T......G.C.C.C.C.T..
CRESTED2      ...T.T......................G..T..........C.T........C.CGC.CC.T.
VULTURINE     ....TCT.A..................G.T............T.......C...G..CC...
```

```
[                  140        150        160        170        180        190      ]
[                   *          *          *          *          *          *       ]
Uganda1         CCATCA-CACTTGACCCTCAACTTCCAAGTCACCATGACCATGAATGGTTACAGGACATACCCTT
Uganda2         ......-.....................................................
Malawi1         ......A.GT...T.............G.........T.....................
Kenya1          ......A..T...T.............G.........T.....................
Angola          .......-T.T...T...-.........G.........T.....................
Reitz           ....A.-T.....T...C.....C.............T.....................C.
Groblershoop    ....A.-T.....T...C....CC...G.........T.....................TC.
Zimbabwe        ....A.-......T........CC...G.C.......T.....................TC.
Namibia1        ....A.-......T........CC.............T.....................TC.
Dundee          ....A.-......T........CC.............T.....................TC.
Namibia2        ....A.-......T........CC.............T.....................TC.
Harrods         .......-T.T...T...-.....................................
Malawi2         .......-T.T...T...-.........G.........T.....................
Underberg       ......A..T...T.............G.........T.....................T...
Zambia          ......A..T...T.............G.........T.....................
Kenya2          ......-..................T.............T.....................
CAR1            ......-T................T.....................................
CAR2            ......-T................T.....................................
CRESTED1        ....A.--..C..CA.TAG...CCT....C....TAC..A.....................TC.
CRESTED2        ....A.--.....TA..AG...CCT..G.C....TAT..A.................G.......
VULTURINE       ....AG--..C.-CT.TA....CCA....C....TA-..T.........A............A.A
```

```
[                200        210        220        230        240        250        260]
[                 *          *          *          *          *          *          *]
Uganda1         AAATCTTATGTTCTTCCTCATTTGGTTATGCTAGACGTACCAGATGGATTTATTGATCGTACACC
Uganda2         ...........................................................
Malawi1         ....TCC.........C...........................................
Kenya1          ....TCC.........C...........................................
Angola          ....TA......................................................
Reitz           ......C...C.................................................
Groblershoop    ......C...C.................................................
Zimbabwe        ............C...C............................................
Namibia1        ..........C..C..............................................
Dundee          ..........C..C..............................................
Namibia2        ............C...............................................
Harrods         ...CTA......................................................
Malawi2         ....TA......................................................
Underberg       ....TCC.........C...........................................
Zambia          ....TCC.........C...........................................
Kenya2          ...........................................................
CAR1            ...........................................................
CAR2            ...........................................................
CRESTED1        ....-AC....AT.A..C..........................................
CRESTED2        ....-AC....AT.A..C.....................T.....................
VULTURINE       ..C.-AC....AT.A..C.........................C.................
```

```
[                  270        280        290        300        310        320  ]
[                   *          *          *          *          *          *  ]
Uganda1        TCACGAGAGATCAGCAACCCCTGCCTGTAATGTCCTATATGACTAGCTTCAGGCCCATTCTT
Uganda2        ..............................................................
Malawi1        ..............................................................
Kenya1         ..............................................................
Angola         ..............................................................
Reitz          ..........................A...................................
Groblershoop   ..........................A...................................
Zimbabwe       ..........................A...................................
Namibia1       ..........................A...................................
Dundee         ..........................A...................................
Namibia2       ..........................A...................................
Harrods        ..............................................................
Malawi2        ..............................................................
Underberg      ..............................................................
Zambia         ..............................................................
Kenya2         ..............................................................
CAR1           ..............................................................
CAR2           ..............................................................
CRESTED1       ..........................GG..................................
CRESTED2       ..............................................................
VULTURINE      ..............................................................
```

Appendix 2.2

Sequence comparisons of 589 base pairs of the 5' end of the mitochondrial DNA cytochrome *b* gene for 13 individuals of *Numida meleagris*. Dots (.) indicate identity with the Central African Republic (CAR) sequence and question marks (?) indicate missing nucleotide data. Guttera = *Guttera pucherani* and Acryllium = *Acryllium vulturinum*.

```
[                  10         20         30         40         50         60     ]
[                   *          *          *          *          *          *     ]
CAR          CTGCAGATACCTCCCTAGCCTTCTCAT?CGTAGCCCACACATGTCGAAATGTCCAATACGGATGA
Dundee       ............................C.............C.....C.............
Namibia2     ............................C.............C.....C.............
Kenya2       ............................?.....................................
Kenya1       ............................C.....................................
Malawi2      ............................C.....................................
Namibia1     ............................?.......?...??????????????????????????
Groblershoop ............................C.............C.....C.............
Harrods      ............................C.....................................
Reitz        ............................C.............C.....C.............
Underberg    ............................C.....................................
Zambia       ............................C.....................................
Zimbabwe     ............................C.............C.....C.............
Guttera      .C.....C................C.CT.............C.....................
Acryllium    .......C.....A.....T.....C.C...G.......TG..C.....C.............

[                70         80         90        100        110        120        130]
[                 *          *          *          *          *          *          *]
CAR          CTAATCCGAAACCTGCACGCAAACGGAGCCTCATTCTTCTTCATCTGCATCTACCTCCACATTGG
Dundee       ..............A..T.....................................................
Namibia2     ..............A..T.....................................................
Kenya2       ............................................................................
Kenya1       ..............A..T.....................................................
Malawi2      ..............A..T.....................................................
Namibia1     ????????????????????????????????????????????????????????????????????
Groblershoop ..............A..T.....................................................
Harrods      ..............A..T.....................................................
Reitz        ..............A..T.....................................................
Underberg    ..............A..T.....................................................
Zambia       ..............A..T.....................................................
Zimbabwe     ..............A..T.....................................................
Guttera      ........C.....A..T...........T........T.....T.....................
Acryllium    ..T...........A.....................................................C..

[                  140        150        160        170        180        190     ]
[                   *          *          *          *          *          *     ]
CAR          CCGAGGCCTATACTACGGCTCCTACCTATATAAAGAAACCTGAAACACAGGAGTAATTCTCCTCC
Dundee       ............................................................................
Namibia2     ............................................................................
Kenya2       ............................................................................
Kenya1       ............................................................................
Malawi2      ............................................................................
Namibia1     ??????????????????..........................................................
Groblershoop ............................................................................
Harrods      ............................................................................
Reitz        ............................................................................
Underberg    ............................................................................
Zambia       ............................................................................
Zimbabwe     ............................................................................
Guttera      .........T..T..T.....A........C..............T...........C.......
Acryllium    .........C...........A.....T..C.....................................
```

```
[              200        210        220        230        240        250        260]
[               *          *          *          *          *          *          *]
CAR            TCACACTAATAGCAACCGCTTTCGTAGGCTACGTTCTTCCTTGAGGCCAAATATCATTCTGAGGG
Dundee         .........................................A..........................
Namibia2       .........................................A..........................
Kenya2         ...........................................................................
Kenya1         .........................................A..........................
Malawi2        .........................................A..........................
Namibia1       .........................................A..........................
Groblershoop   .........................................A..........................
Harrods        .........................................A..........................
Reitz          .........................................A..........................
Underberg      .........................................A..........................
Zambia         .........................................A..........................
Zimbabwe       .........................................A..........................
Guttera        ............T.....C..T...........A.....A.............C.........
Acryllium      .......G.....C.....C.............A..C..A.......................A
```

```
[                270        280        290        300        310        320      ]
[                 *          *          *          *          *          *       ]
CAR            GCTACTGTCATTACTAATCTATTCTCAGCTATCCCCTACATTGGACAAACTCTAGTAGAGTGGGC
Dundee         ...........................................................................
Namibia2       ...........................................................................
Kenya2         ...........................................................................
Kenya1         ...........................................................................
Malawi2        ...........................................................................
Namibia1       ...........................................................................
Groblershoop   ...........................................................................
Harrods        ...........................................................................
Reitz          ...........................................................................
Underberg      ...........................................................................
Zambia         ...........................................................................
Zimbabwe       ...........................................................................
Guttera        .....C.....C..C..................T........C......G.CT.G.......A..
Acryllium      ..G..C.....C..C.........T........T..T..............CT.......A..A..
```

```
[              330        340        350        360        370        380        390]
[               *          *          *          *          *          *          *]
CAR            GTGGGGAGGGTTTTCAGTCGACAACCCCACCCTCACTCGATTTTTCGCCCTACACTTCCTTCTCC
Dundee         ...........................................................................
Namibia2       ...........................................................................
Kenya2         ...........................................................................
Kenya1         ...........................................................................
Malawi2        ...........................................................................
Namibia1       ...........................................................................
Groblershoop   ...........................................................................
Harrods        ...........................................................................
Reitz          ...........................................................................
Underberg      ...........................................................................
Zambia         ...........................................................................
Zimbabwe       ...........................................................................
Guttera        ...A.....A..C..T...........A.....T..C.....C..T..............C....
Acryllium      ..................T..T.....A........C.....C..................C....
```

```
[                  400        410        420        430        440        450     ]
[                   *          *          *          *          *          *      ]
CAR          CCTTCGTCATCGCAGGAATCACAATTATCCACCTCACATTCCTTCACGAATCGG?CTCAAACAAC
Dundee       ..........................................................G.........
Namibia2     ..........................................................G.........
Kenya2       ..........................................................G.........
Kenya1       ..........................................................G.........
Malawi2      ..........................T...............................G.........
Namibia1     ............??????????????????????????????????????????????????????
Groblershoop ..........................................................G.........
Harrods      ..........................................................G.........
Reitz        ..........................................................G.........
Underberg    ..........................................................G.........
Zambia       ..........................................................G.........
Zimbabwe     ..........................................................G.........
Guttera      ....TA............T..C..........T..................A.G.........
Acryllium    ......C...........T..C..C.......................A.G.........
```

```
[                460        470        480        490        500        510        520]
[                 *          *          *          *          *          *          *]
CAR          CCCCTAGGCATT?CATCCGACTCAGACAAAATCCCATTCCACCCCTACTACTCCATCAAGAA??T
Dundee       ............T.....................................................AG.CA.
Namibia2     ............T.....................................................AG.CA.
Kenya2       ............T.....A...............................................AG.CA.
Kenya1       ............T.....................................................AG.CA.
Malawi2      ............T.....A...............................................AG.CA.
Namibia1     ????????????????.....................................................AG.CA.
Groblershoop ............T.....................................................AG.TA.
Harrods      ............T.....A...............................................AG.CA.
Reitz        ............T.....................................................AG.TA.
Underberg    ............T.....................................................AG.CA.
Zambia       ............T.....................................................AG.CA.
Zimbabwe     ............T.....................................................AG.CA.
Guttera      ............T.....A.........................A..............G.TA.
Acryllium    ............A....AA.......T.....................................AG.CA.
```

```
[                  530        540        550        560        570        580     ]
[                   *          *          *          *          *          *      ]
CAR          CCTAGGCCTAACACTTATACTCACCCCACTCCTAACCCTAGCCCTATTCTCCCCAAACCTACTAG
Dundee       ..........................................................................
Namibia2     ..........................................................................
Kenya2       ..........................................................................
Kenya1       ..........................................................................
Malawi2      ..........................................................................
Namibia1     ..........................................................................
Groblershoop ........................T.................................................
Harrods      ..........................................................................
Reitz        ........................T.................................................
Underberg    ..........................................................................
Zambia       ..........................................................................
Zimbabwe     ..........................................................................
Guttera      ..............C..G........C.....G......A...................C....
Acryllium    .........GGT...C...........C........AT..A...................T....
```

```
[                  ]
[                  ]
CAR             GTGA
Dundee          ....
Namibia2        ....
Kenya2          ....
Kenya1          ....
Malawi2         ....
Namibia1        ....
Groblershoop    ....
Harrods         ....
Reitz           ....
Underberg       ....
Zambia          ....
Zimbabwe        ....
Guttera         .A..
Acryllium       ....
```

Appendix 3

Sequence comparisons of 321 base pairs of the 5' end of the mitochondrial DNA control region for 27 haplotypes identified within *Numida meleagris coronata* and *N. m. damarensis* (H19, H23 & H24) from southern Africa. Dots (.) indicate identity with the haplotype H1 sequence and question marks (?) indicate missing nucleotide data. CAR159 = the outgroup from the Central African Republic.

```
[               10         20         30         40         50         60         70]
[                *          *          *          *          *          *          *]
H1      TTATGGTACCGGTACTATATACTATATATGTACTAAGCCCATATATATGTAAACGGACATAAACACCTCC
H2      ..........................................................................
H3      ..........................................................................
H4      ..........................................................................
H5      ..........................................................................
H6      .........................................................................T
H7      ..........................................................................
H8      ..........................................................................
H9      ..........................................................................
H10     ..........................................................................
H11     ..........................................................................
H12     ..........................................................................
H13     ..........................................................................
H14     ..........................C.................................................
H15     ..........................................................................
H16     ..........................................................................
H17     ..........................................................................
H18     ..........................................................................
H19     ..........................................................................
H20     ....................T.............A.....................................
H21     ....................................A.....................................
H22     ....................................A.....................................
H23     ..........................................................................
H24     ..........................................................................
H25     ..........................................................................
H26     ..........................................................................
H27     ..........................................................................
CAR159  ..........A.................?A......A.............................T......
```

```
[              80        90       100       110       120       130       140]
[               *         *         *         *         *         *         *]
H1      TCCCCATTCCTCCCAAATGTACTAAAACGTGTAATGCTCCTAGACATAAACTATAATCTACCATAATACT
H2      ...............................................................................
H3      ...............................................................................
H4      ...............................................................................
H5      ...................................T...........................................
H6      ...............................................................................
H7      ...............................................................................
H8      ..............................................................G................
H9      ...............................................................................
H10     ...............................................................................
H11     ...............................................................C...............
H12     ...............................................................C.........C...
H13     ...............................................................C....C.........C...
H14     ...............................................................C....C.........C...
H15     ...............................................................C.........C...
H16     ...............................................................C.........C...
H17     ...............................................................C.........C...
H18     ...............................................................C.........C...
H19     ...............................................................C.........C...
H20     ...............................................................C.........C...
H21     ...................................T...........................C.........C...
H22     ...................................T...........................C.........C...
H23     ...............................................................C.........C...
H24     ..........................................................G.....CT........C...
H25     ...............................................................CT........C...
H26     ...............................................................CT........C...
H27     ...............................................................CT........C...
CAR159  A.........................A.........T.C...........C...G..C.....C.....

[              150       160       170       180       190       200       210]
[               *         *         *         *         *         *         *]
H1      TGTCCCCCAACTCCCAAGTCACCATGATCATGAATGGTTACAGGACATACCTCTAAATCTCATGCTCTTC
H2      ...............................................................C...............
H3      ......T........................................................................
H4      ..........................C.....................................................
H5      ..........................C.....................................................
H6      ...............................................................CT..............
H7      ..........C....................................................................
H8      ..........C...G................................................................
H9      ..........C.....................................................T.........
H10     ..........C.....................................................T..............
H11     ..........C.....................................................T..............
H12     ......T....C....G..............................................T.........
H13     ..........C.....................................................T.......T......C..
H14     ..........C.................C...................................................C..
H15     ......T....C....G..............................................T.......C..
H16     ......T....C..?.G..............................................T.......C..
H17     ......T....C....G..............................................T...T..C..
H18     ......T....C....G.C............................................T...T..C..
H19     ......T....C...................................................T.......C..
H20     ......T....C...................................................T.......C..
H21     ......T....C...................................................T.......C..
H22     ....T.T....C...................................................T.......C..
H23     ......T....C.................C.................................T...T..C..
H24     ......T....C...................................................C..
H25     C.....T....C......C............................................T.......C..
H26     C.....T....C......C............................................T...T..C..
H27     ......T....C......C.....................................CT.......T.........
CAR159  ..A...T.....TT.............C.....................CT.......T...T.....
```

```
[              220        230        240        250        260        270        280]
[               *          *          *          *          *          *          *]
H1       CTCATTTGGTTATGCTAGACGTACCAGATGGATTTATTGATCGTACACCTCACGAGAGATCAGCAACCCC
H2       ..........................................................................
H3       ..........................................................................
H4       ..........................................................................
H5       ..........................................................................
H6       ..........................................................................
H7       ..........................................................................
H8       ..........................................................................
H9       ..........................................................................
H10      .C........................................................................
H11      .C........................................................................
H12      ..........................................................................
H13      ..........................................................................
H14      ..........................................................................
H15      ..........................................................................
H16      ..........................................................................
H17      ..........................................................................
H18      .C........................................................................
H19      ..........................................................................
H20      ..........................................................................
H21      ..........................................................................
H22      ..........................................................................
H23      ..........................................................................
H24      ..........................................................................
H25      ..........................................................................
H26      ..........................................................................
H27      ..........................................................................
CAR159   ..........................................................................

[              290        300        310        320 ]
[               *          *          *          * ]
H1       TGCCTATAATGTCCTATATGACTAGCTTCAGGCCCATTCTT
H2       ........................................
H3       ........................................
H4       ........................................
H5       ........................................
H6       ........................................
H7       ........................................
H8       ........................................
H9       ........................................
H10      ........................................
H11      ........................................
H12      ........................................
H13      ........................................
H14      ........................................
H15      ........................................
H16      ........................................
H17      ........................................
H18      ........................................
H19      ........................................
H20      ........................................
H21      ........................................
H22      ........................................
H23      ........................................
H24      ........................................
H25      ........................................
H26      ........................................
H27      ........................................
CAR159   .....G..................................
```